

**Development of a novel electrochemical assay for the rapid detection of
pathogenic fungi and identification of clinically relevant *Candida*
species.**

Volume 1 of 1

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Abstract

A number of fungal species, particularly *Candida* species, are opportunistic pathogens capable of causing disease in humans. These range from relatively mild infections in healthy individuals (e.g. thrush) to life threatening systemic infections and colonisation of major organs in immunocompromised individuals. Existing methods of identifying *Candida* species in clinical samples are time and resource intensive and are not always specific enough to differentiate between drug-susceptible and drug-resistant species.

Atlas Genetics have developed a novel electrochemical assay for the detection of nucleic acid from target pathogens using PCR followed by hybridisation by labelled probes. The work describes the development of a suite of probes and optimisation of reaction conditions for the rapid detection of fungal pathogens using this assay. A suite of five species-specific probes was developed to detect the five most clinically relevant *Candida* species as well as a pan-fungal probe capable of detection of DNA from any fungal species. Additionally, since the assay was novel, the work investigated other aspects such as probe and primer design which led to the development of a quick bioinformatics approach for design of species-specific probes which is also described. The results demonstrated that species-specific detection of *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* was possible, and that there was no cross reactivity exhibited by any probe with isolates from a test panel of other *Candida* species. The limit of detection of the assay was shown to be approximately one genome in 1ml of blood or 10 whole cells in 1ml of blood.

The use of solid-state electrodes for detection provides an opportunity for miniaturisation of the assay into a robust, easily operated, portable system capable of rapid detection of pathogenic DNA in clinical samples either at point of care or in the microbiological laboratory.

List of Abbreviations.

BLAST – Basic Local Alignment Search Tool

BSC – biological safety cabinet

CBS – Centraal Bureau voor Schimmelcultures

DPV – differential pulse voltammetry

dsDNA – double-stranded DNA

IFI – invasive fungal infection

ITS – internal transcribed spacer

PCR – polymerase chain reaction

qPCR – quantitative polymerase chain reaction

rRNA – ribosomal RNA

ssDNA – single-stranded DNA

T7 exonuclease - dsDNA-specific gene 6 exonuclease from bacteriophage T7

UV – ultra-violet

Chapter 1. Introduction.

1.1 Fungal infections

Fungal species are capable of causing myriad human diseases which range from relatively trivial topical infections such as athlete's foot and thrush, to life threatening invasive conditions such as fungaemia (fungal cells present in the blood) which can lead to infection of major organs. A wide range of fungi are capable of causing invasive fungal infections and these include, but are not limited to, species of the genera *Candida*, *Aspergillus*, *Cryptococcus* and *Saccharomyces*. Invasive fungal infections (IFI) are a considerable burden on healthcare resources. A study by Wilson *et al.* (2002) estimated that in the US, for the year 1998, the cost of the first year of treatment for fungal infections came to \$2.6 billion nationally, which represented 0.24% of the total US health expenditure. The average cost per person with a fungal infection was estimated at \$31,200, which was nearly eight times higher than the average amount spent on healthcare by the rest of the population. At least some of the considerable cost of hospitalisation and inpatient care which made up this figure was likely to have been due to treatment of the underlying disease which predisposed the patient to IFI. Nonetheless, it cannot be ignored that IFI necessitates continued hospital care and treatment at no small cost. A more recent study performed by Zaoutis *et al.* (2005) estimated that in the United States cases of candidaemia in adults accounted for increased hospital costs of between \$33,604 and \$45,602 per episode for the year of 2000. For the same period the increased costs for paediatric patients were estimated at between \$65,058 and \$119,474.

Invasive fungal infections, although rare, are most commonly associated with individuals with a weakened immune system due to underlying disease, either because of the progression of the disease itself or the treatment that is administered to the patient. They are particularly associated with patients suffering from cancer, HIV infections and patients who have received organ transplants (Jarvis, 1995; Wenzel, 1995) - HIV patients because of the action of the virus on lymphocytes, cancer patients due to the cytotoxic treatment they receive, and transplantation patients due to immunosuppressant drugs

with which they are treated. As a result these patients require lengthy care in hospitals and invasive fungal infections are frequently nosocomially acquired (Wisplinghoff *et al.*, 2004; Costa-de-Oliveira *et al.*, 2008). Over the last few decades the immunocompromised patient population has increased due to advances in medical treatment and it is no coincidence that the incidence of invasive fungal infections has also increased. For example, in the United States the incidence of sepsis caused by fungal organisms increased by 207% from 5,231 cases in 1979 to 16,042 cases in 2000 (Martin *et al.* 2003); Arendrup *et al.* (2008) reported an increase in fungaemia in Denmark of 17% over the two years between 2004 and 2006, and Abelson *et al.* (2005) reported a significant increase in episodes of fungaemia in a children's hospital in the United States over the 11-year period from 1991 through 2001.

Of the fungi implicated in invasive infections, species of the genus *Candida* are by far the most prevalent in the clinical setting. They are members of the hemiascomycetes group of fungi and reproduce vegetatively by multipolar budding, forming blastoconidia which may form branching chains. They are also capable of forming well developed pseudohyphae (McGinnis, 1980). Some species (*C. albicans* and *C. dubliniensis*) are capable of producing a structure called a germ tube which is an immature hypha and as such is a known virulence factor (Calderone & Fonzi, 2001) though it can be used for identification of these species from clinical samples. *Candida* species, though frequently associated with mucosal membranes of humans and other animals are ubiquitous in the environment, with isolates from the five clinically relevant species having been found in a range of environments, according to the Centraalbureau voor Schimmelcultures (CBS) yeast database: on plants, in tree bark, in soil, in the gut of insects, in fermentations of various substrates, in foods such as yoghurt and on rotting fruit and vegetation. They are capable of causing invasive disease by endogenous colonisation following damage to the skin or mucosa or by direct access to the circulation by, for example, surgery or introduction of a central venous catheter (Davey, 2010).

Candida species are responsible for 70-80% of diagnosed fungal bloodstream infections in the United States (Beck-Sague and Jarvis, 1993; Fridkin and

Jarvis, 1996; Trick *et al.*, 2002) and they are a leading cause of nosocomial infections in patients undergoing lengthy hospital treatments (Pfaller, 1996) accounting for 7.6% of nosocomially acquired bloodstream infections in the United States and ranking them as the fourth most commonly encountered microbe isolated from blood (Edmond *et al.*, 1999). They are frequently encountered in patients with other complications who develop IFI, e.g. following solid organ transplant (Neofytos *et al.*, 2010) and those who are critically ill (Vazquez, 2010). Throughout the majority of the world *C. albicans* is the most commonly isolated species from patients with candidaemia, accounting for approximately 45-65% of cases while four other species - *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* – collectively account for a further 35-50% of infections (Hajjeh *et al.*, 2004; Tortorano *et al.*, 2004; Pfaller *et al.*, 2010). The incidences of the species can vary with patient factors such as age - infections caused by *C. glabrata* are more common in adult patients while *C. parapsilosis* is considerably more common in the paediatric patient population (Pfaller & Diekema, 2002). This is due to the frequent handling of premature neonates by healthcare workers which allows the spread of the yeast. Additionally, *C. parapsilosis* has been associated with biofilm production linked to indwelling lines (Shin *et al.*, 2002) which are used to provide parenteral nutrition to premature neonates. Geographical location can also affect the incidence of species. In countries with a warmer climate, such as in Asia and S. America, the proportions of the above species frequently differ (Tan *et al.*, 2009) and some studies have reported that the incidence of *C. tropicalis* in these countries was higher than that of *C. albicans* making *C. tropicalis* the leading causative agent of invasive infections (Chakrabarti *et al.*, 2009; Pereira *et al.*, 2010) although this was not a general trend in these areas (Bruder-Nascimento *et al.*, 2010). The remaining small percentage of candidaemia cases are caused by a number of less frequently encountered *Candida* species such as *C. lusitanae* and *C. guilliermondii* (Kao *et al.*, 1999; Health Protection Agency, 2006).

The incidence of candidaemia reported in the population is relatively small with estimates of between 1.9 - 11 cases per 100,000 inhabitants being reported for European countries (Tortorano *et al.*, 2006). The upper limit of

this range was recorded in Denmark and was anomalously high, being three-fold higher than the next highest incidence. In the United States the incidence of candidaemia is generally higher than that of European countries: the study by Hajjeh *et al.* (2004) found an incidence of 24 cases per 100,000 inhabitants in Baltimore and a study by Kao *et al.* (1999) reported incidences of 8.7 and 7.1 cases per 100,000 inhabitants in Atlanta and San Francisco respectively. The incidence of invasive *Candida* infections (which includes candidaemia), was estimated at between 22 to 29 cases per 100,000 inhabitants recorded over an 8-year period (Pfaller & Diekema, 2007). The disease, therefore, is not very common. However, the immunocompromised status associated with the majority of patients who develop candidaemia reduce the chances of survival and the mortality of candidaemia is high with estimates of crude mortality ranging between 30% - 50% (Tortorano *et al.*, 2004; Horn *et al.*, 2007) and so the problem, whilst relatively rare, is deadly serious.

1.2 Treatment of invasive fungal infections

There are a number of antifungal drugs available for treatment of fungaemia and some of the most clinically relevant are discussed here. Fluconazole is a triazole drug which became available in the early 1990's and is now commonly used as the frontline drug for invasive fungal infections. Like the other azole drugs, it exercises its antifungal effects by preventing synthesis of the predominant fungal cell membrane component ergosterol by inhibiting cytochrome P-450-dependent 14 α -sterol demethylase (Hitchcock *et al.*, 1990). Fluconazole has broad activity across yeasts and is less toxic and less expensive than some other antifungals. Its main disadvantage is that several *Candida* species, most importantly *C. krusei* and *C. glabrata* exhibit resistance to it. *C. krusei* is inherently resistant to fluconazole due to possession of a modified 14 α -sterol demethylase enzyme which is less susceptible to inhibition by fluconazole (Orozco *et al.*, 1998) while resistance in *C. glabrata* is mediated by an upregulation of the ATP-binding cassette transporters Cdr1p and Pdh1p which actively efflux fluconazole from the fungal cells (Vermitsky & Edlind, 2004). The drug is also ineffective against infections due to filamentous fungi.

Other triazole compounds have been developed which overcome some of the limitations exhibited by fluconazole. These include voriconazole, itraconazole and the more recently derived posaconazole. Voriconazole is recommended as alternative therapy for the treatment of invasive infections caused by *C. krusei* and fluconazole-resistant, voriconazole susceptible *C. glabrata* (Pappas *et al.*, 2009; Pfaller *et al.*, 2010b). It also has a wider range of targets, being active against dimorphic fungi and filamentous fungi as well as yeasts (Fera *et al.*, 2009) and is cheaper than the alternative antifungals amphotericin B and the echinocandins, caspofungin and anidulafungin, making it a more favourable treatment option. Itraconazole is a choice antifungal agent for use against mucosal *Candida* infections and as antifungal prophylaxis for patients at risk of developing invasive fungal infections (Korting & Schöllmann, 2009; Pappas *et al.*, 2009). However, reduced susceptibility to this drug *in vitro* has been exhibited by isolates of *C. glabrata* and *C. krusei* (Messer *et al.*, 2009). Posaconazole, like voriconazole, exhibits improved efficacy against *C. glabrata* and *C. krusei* isolates compared to fluconazole *in vitro*, (Messer *et al.*, 2009) and has shown promise in prophylactic therapy for patients at risk from invasive fungal infections (Cornely *et al.*, 2007, Ullmann *et al.*, 2007) and as salvage therapy for patients with invasive fungal infections which were refractory to conventional treatment (Walsh *et al.*, 2007).

Amphotericin B (AmB) was the mainstay drug for fungal infections for several decades and it is still a useful drug today, with a broad range of activity, which includes both yeasts and filamentous fungi. Although it is relatively cheap its use is limited by its side effects which include nephrotoxicity, fevers and hypertension (Enoch *et al.*, 2006). A less toxic liposomal formulation is available although it is considerably more expensive. Its antifungal action is due to molecules of the drug interacting directly with fungal cell membrane sterols to form pores (Holz, 1974) which alter the membrane's permeability, allowing the leakage of vital cytoplasmic components from the fungal cell leading to its demise (Ghannoum & Rice, 1999). Resistance to AmB is not common and *C. lusitanae* is the most clinically relevant species in this respect. As a result AmB is recommended to treat patients with *Candida*

infections caused by species such as *C. glabrata* or *C. krusei* which are frequently resistant to fluconazole, or for the treatment of infections caused by filamentous fungi.

Flucytosine, or 5-fluorocytosine, is one of the oldest antifungals and is still used in combination therapy, frequently with amphotericin B, in certain instances of IFI (Bennett *et al.*, 1979). It does not possess antifungal properties until it is taken up into the fungal cell where its conversion into 5-fluorouracil and 5-fluorodeoxyuridine inhibits synthesis of RNA and DNA (Waldorf & Polak, 1983). It is of particular use when meningeal involvement is suspected as it readily crosses the blood/brain barrier into the cerebrospinal fluid (CSF) (Vermes *et al.*, 2000).

The echinocandins are a group of antifungal agents which include caspofungin and micafungin, and work by inhibiting the cell-wall enzyme complex β -1,3-D-glucan synthase which causes damage to the fungal cell wall leading to a fungicidal effect in *Candida* (Denning, 2003). Echinocandins are not associated with serious side effects as mammalian cells lack any orthologous target molecule, they have few drug interactions and species-specific resistance has not been widely reported. As it is a more aggressive antifungal than fluconazole its use is recommended in cases involving fungal infections in neutropenic patients or as an alternative treatment for non-neutropenic patients presenting with fluconazole-resistant *Candida* species (Pappas *et al.*, 2009). Table 1.1 summarises the antifungal resistance profiles of the most clinically relevant *Candida* species to the compounds described above.

There are many considerations that must be made when choosing the appropriate treatment for invasive fungal infection. The most important of these regard the health and safety of the patient but the cost of treatment must be considered also. If inadequate therapy is administered, such as inadequate dosing of an antifungal or use of an inappropriate antifungal compound, it will increase the chance of failure of therapy and in the worst case the patient may die. Even if the patient survives the time to recovery will

Table 1.1. Resistance/susceptibility patterns observed in common *Candida* species to a range of antifungal agents (taken from Pappas *et al.*, 2009).

<i>Candida</i> species	Fluconazole	Voriconazole	Amphotericin B	Candins
<i>C. albicans</i>	S	S	S	S
<i>C. tropicalis</i>	S	S	S	S
<i>C. parapsilosis</i>	S	S	S	S to R*
<i>C. glabrata</i>	S-DD to R	S-DD to R	S to I	S
<i>C. krusei</i>	R	S	S to I	S

S – susceptible, S-DD – susceptible dose dependent, I – intermediately resistant, R – resistant

*Echinocandin resistance among *C. parapsilosis* isolates is uncommon

be greatly increased as long as they are treated inadequately and so the cost of treatment and hospital care will increase correspondingly. Inadequate therapy is often due to treatment of an invasive fungal infection with an antifungal drug to which the infecting fungus is resistant (Garey *et al.*, 2007). As mentioned above this is generally a species-specific phenomena and rapid identification of the causative organism to the species level is essential to ensure that effective treatment is administered as soon as possible.

1.3. Detection of fungal pathogens

Rapid diagnosis and identification of fungal pathogens to the species level is essential for successful treatment of invasive fungal infections. The difficulty in diagnosing fungal infections lies in the fact that there are rarely symptoms that definitively point to an invasive fungal infection and diagnoses are frequently made on clinical suspicion based on risk factors which increase the likelihood that a patient will develop an invasive *Candida* infection, such as immunosuppression, presence of an intravenous catheter, use of broad-spectrum antibiotics and trauma to the gastrointestinal tract (Ostrosky-Zeichner *et al.*, 2007; Hsueh *et al.*, 2009). Pre-emptive diagnosis, therefore, is ultimately based on a judgement call which may still be incorrect even with the weight of evidence supporting the decision.

1.3.1 Culture-based diagnostic methods

A definitive diagnosis of invasive fungal disease can be made following isolation of fungal colonies from clinical samples such as blood cultures or

biopsy specimens. Following isolation, further tests must be performed on the fungus to confirm its identity, at which point any changes to provide the patient a more appropriate antifungal treatment regime can be made. There are two major problems with this 'gold standard' approach. The first is the time to diagnosis; a study by Morris *et al.* (1996) found that it took between 2-3 days for 50% of the cultures tested to become positive and some required two weeks before recovery of fungal cultures. The time to species identification is further increased by the need to perform biochemical and/or morphological tests to accurately identify the organism. Germ tube testing is a relatively quick test which can be performed immediately after recovery of a positive yeast blood culture (Sheppard *et al.*, 2008). It requires a reasonable degree of expertise to perform accurately, however, and can only positively identify *C. albicans*. Chromogenic media such as CHROMagar and Chromogenic *Candida* agar are available to differentiate yeast species based on the colour of the colonies. This method has high sensitivity and specificity for the target species, is relatively inexpensive and requires little additional expertise but still requires at least 48hrs to achieve accurate identification and only a few species can be differentiated in this manner (Odds & Bernaerts, 1994; Ghelardi *et al.*, 2008). Additionally, misidentification is possible due to the existence of a large number of yeast species, some of which can produce colonies of similar colours and morphologies to the target species (Hospenthal *et al.*, 2006). More advanced biochemical tests can be performed on cultured yeasts, such as the API Candida and AUXACOLOR systems. These tests can distinguish a much greater range of species but require a degree of expertise to perform and require a further 24 - 48hrs to make species identifications (Campbell *et al.*, 1999). The second problem is the issue of sensitivity – estimates have suggested that ~50% of blood cultures from patients with candidaemia prove negative for *Candida* species (Pemán & Zaragoza, 2009). The outcome of these limitations is that effective therapy is likely to be delayed and this can have a significant impact on mortality (Morrell *et al.*, 2005; Garey *et al.*, 2006).

In light of these limitations the moniker of 'gold standard' is perhaps not well deserved by culture based methods of fungal detection and identification in

the clinical setting. To address this issue, numerous non-culture based tests have been developed for detection and identification of fungal species. They can offer improved specificity and sensitivity and, crucially, decreased time to diagnosis. The tests are, broadly speaking, of two types: those that identify fungi based on serological means such as antibody or antigen detection and those that detect nucleic acid.

1.3.2 Serological methods

Serological tests for detection of invasive fungal infections based on antibody or antigen detection have been in existence for decades. Antigen detection has utilised cell wall components such as mannan in the case of *Candida* species and galactomannan for *Aspergillus* species. Some of the earliest tests, such as Pastorex-*Candida* and Cand-Tec, used latex agglutination for detection of mannan but such tests exhibited very poor sensitivity (Herent *et al.*, 1992; White *et al.*, 2005). Newer serological methods have made use of enzyme immunoassays for detection of both the mannan antigen and anti-mannan antibodies e.g. Platelia *Candida* Antibody and Platelia *Candida* Antigen tests. These are an improvement over latex agglutination with high specificity and improved, though still relatively low, sensitivity. It was shown that the sensitivity could be increased by combining the results of both antibody and antigen tests together (Sendid *et al.*, 1999) but the requirement for two tests complicates the process and increases the cost.

A major drawback to these methods is the inability to directly make a specific identification to species level. Therefore, while serological techniques allow some time to be saved due to their ability to detect fungi directly from prepared blood samples this saving is somewhat offset by the need to perform further biochemical and morphological tests to make species level identification to ensure appropriate treatment is being administered.

1.3.3 Nucleic acid detection methods

Detection of nucleic acid offers significant advantages over other methods. The polymerase chain reaction (PCR) allows amplification of the

smallest amounts of target nucleic acid to quantities which can be easily detected either directly (e.g. qPCR, real-time PCR) or by a number of downstream techniques (e.g. ELISA, pyrosequencing) in a matter of hours. Most importantly, however, is the presence of species-specific regions of nucleic acid which allows for the design of assays capable of identification to the species level. The simplest methods involve using PCR for direct identification of fungi by amplifying products of different sizes which are specific for the species of interest (e.g. Kanbe *et al.*, 2002; Arancia *et al.*, 2009) or generating a species-specific pattern of products as in random amplification of polymorphic DNA (Bautista-Muñoz *et al.*, 2003). More complex methods have involved using radiolabelled species-specific probes to detect fungal DNA following Southern blotting of PCR products (van Deventer *et al.*, 1995), digoxigenin-labelled probes in enzyme immunoassays (Fujita *et al.*, 1995; Shin *et al.*, 1997). The above methods require post-PCR sample handling but due to the rapidity of PCR-based methods rapid detection and identification of fungi can still be achieved. However, such handling increases the potential for contamination of the samples leading to false positive results.

More recently a large number of techniques using fluorescent probes in real-time and qPCR assays (e.g. Maaroufi *et al.*, 2003; Metwally *et al.*, 2007; Hata *et al.*, 2008; Lau *et al.*, 2008) have been described. These methods all share the advantage that detection occurs during the PCR amplification itself removing the need for post-PCR handling and so allowing even more rapid identification. The methods which make use of fluorescently labelled probes, e.g. Taqman, can achieve specific detection at the level of PCR primers and also the probe molecules and this allows for the detection of a greater range of species. Furthermore, some of these tests are capable of detection of fungal DNA from prepared blood samples without enrichment. When combined with the species-level identification which is possible during real-time PCR it allows for considerable reduction in time to diagnosis. Several methods capable of direct fungal detection from blood have been described (e.g. McMullan *et al.*, 2008; Zhao *et al.*, 2009) and in combination with real-time PCR it allows for the design of extremely rapid assays (<3 hours).

DNA sequencing is the most reliable molecular technique for definitive identification of fungal species. Traditional sequencing using di-deoxy termination has been used to identify clinically relevant yeasts (Ciardo *et al.*, 2006; Linton *et al.*, 2007) and is advantageous as it usually produces an unambiguous output which can quickly be compared to public or in house sequence databases to identify a species. This characteristic is particularly useful when attempting to identify species exhibiting characteristics which are not covered by a biochemical test panel or a species which has not been implicated in human disease before (Borman *et al.*, 2008). The method is relatively expensive to perform, however, and relies on the presence of matching sequence data in databases to make identifications. Pyrosequencing is an advance on di-deoxy termination sequencing and is cheaper to perform but produces much shorter reads. As a result the target for DNA sequencing must be carefully chosen to ensure sufficient variability for identification of fungal species. Montero *et al.* (2008) compared pyrosequencing to di-deoxy sequencing and found that pyrosequencing correctly identified 69.1% of 133 isolates compared to 78.9% identified by di-deoxy sequencing. All isolates of the most clinically relevant fungi were successfully identified by pyrosequencing. The method has been shown to be capable of identification of very closely related isolates by use of an appropriate target sequence, Borman *et al.* (2009) reported the successful differentiation of the three species of the *C. parapsilosis* species complex, *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*, using target sequence within the second internal transcribed spacer (ITS2) region.

Molecular detection of fungi is undoubtedly quicker, particularly the PCR-based tests, and requires less expertise to interpret compared with conventional tests based on morphology and biochemical testing but they still require a well resourced laboratory with trained personnel. In the absence of this, patient samples must be sent to such facilities for testing and the results communicated back to the hospital, and so valuable time is wasted even with the rapid nucleic acid detection methods. A solution would be the incorporation of a nucleic acid based test into an automated system that could

be used at or near the point of care with minimal need for training or interpretation by the user. However, fluorescence-based techniques require sophisticated optical components that are difficult to miniaturise so alternative methods for detection would have to be found to allow the development of such a device.

1.4. Nucleic acid detection using electrochemically labelled probes.

Hillier *et al.* (2004a) demonstrated electrochemical detection of DNA using oligonucleotide probes labelled at the terminal 5' nucleotide with the electroactive compound ferrocene. This ferrocene-labelled oligonucleotide molecule was shown to be digested by S1 nuclease to liberate labelled mononucleotides and short oligonucleotides which could be detected at the surface of a solid state electrode using differential pulse voltammetry (DPV). Briefly, a sweep of increasing voltage from -100mV to +500mV is performed and, at a voltage determined by the composition of the ferrocene molecule, electron transfer from the ferrocene to the electrode occurs, producing a measurable current. DPV is used as it allows greater sensitivity due to the separation of the capacitive current (caused by charged molecules aligned at the surface of the electrode and the electrolyte solution) from the Faradaic current (caused by electron transfer due to chemical reactions taking place). The larger the amount of ferrocene-nucleotide that is present at the electrode surface, the higher the current produced. Undigested probe was found to produce a substantially lower current compared to the digested ferrocene-mononucleotide possibly due to a difference in the diffusion coefficient of undigested oligonucleotide, hindering its diffusion to the electrode surface, or by inhibition of electron transfer due to the presence of the additional atoms present in an intact oligonucleotide molecule. The work was extended by Hillier *et al.* (2004b) to demonstrate detection of target DNA using a ferrocene-labelled probe. A short, synthetic target molecule was allowed to hybridise to a complementary ferrocene-labelled probe molecule and digestion of the probe:target heteroduplex was achieved by using the double-strand DNA (dsDNA)-specific gene 6 exonuclease from bacteriophage T7 (T7 exonuclease; Kerr & Sadowski, 1972) resulting in the release of ferrocene-

mononucleotides which were detected on solid electrodes using DPV, as above.

The procedure was developed further by Atlas Genetics for the purpose of designing a rapid assay for the detection of DNA from pathogenic organisms. A PCR step was incorporated to amplify target DNA followed by post-PCR addition of a ferrocene-labelled oligonucleotide probe targeted to the region which was amplified. The single strand DNA (ssDNA) probe could then bind to the amplified target site either by displacement of the non-target strand of the dsDNA amplicon or by binding to asymmetrically produced ssDNA amplicon and digestion of the hybrid molecule was achieved using T7 exonuclease as above. The products of the exonuclease digestion could then be assayed using DPV measurements on screen-printed carbon electrodes and the current produced by the oxidation of released ferrocene-mononucleotides measured.

Using this approach, preliminary assays demonstrated successful detection of DNA from *Neisseria meningitidis* while non-target DNA or DNA-free samples gave a consistently low, background level of current (S. Boundy, unpublished data). The test demonstrated proof of concept of the electrochemical assay for the detection of DNA from target species. The use of solid-state electrodes was a key feature as it would allow miniaturisation of the assay and Atlas Genetics have proposed the idea of designing a portable system incorporating components which would allow an assay to be automated and performed at or near the point of care with minimal input and interpretation by the user.

1.4.1 Electrochemical detection of fungi

The at-or-near point of care electrochemical method of DNA detection proposed by Atlas Genetics has a number of potential advantages which make it particularly suitable for detection of fungal pathogens. As with other PCR-based methods it is potentially capable of identification to species level and multiplex detection of several pathogens at once is possible. The use of short target sequences for amplification (<500bp) combined with a low

reaction volume suitable for manipulation by microfluidics decreases the time required to complete PCR and, if combined with an appropriate platform capable of rapid temperature ramping, would permit extremely rapid PCR. The reagents for DNA extraction, PCR amplification, probe hybridisation and exonuclease digestion would be present on disposable, single-use test cards. This would reduce the possibility of sample contamination and, combined with the use of solid electrodes for detection, would allow the detection and interpretation steps of the assay to be completely automated and require minimal expertise on the part of the user. Hence the assay could allow a species-specific identification of a fungal pathogen potentially within an hour and appropriate treatment could be administered at once. The main difficulty associated with such a device is the consistent recovery of fungal DNA from the assayed clinical sample, since fungal titres can be very low (Loeffler *et al.*, 2000).

Before any assay can be miniaturised and performed on Atlas Genetics' proposed Velox™ instrument it must first be developed on the benchtop and demonstrated to be capable of detection of DNA from target organisms as well as having a satisfactory degree of specificity and sensitivity.

1.5 Aim

The aim of the project was to develop a benchtop assay to allow electrochemical detection of DNA from *Candida* species on solid electrodes utilising Atlas Genetics ferrocene-labelled oligonucleotide approach. For the initial probe design the questions of which species to identify and the region of the genome to be targeted had to be considered, as well as the possibility of including probes for more generalised detection (i.e. species specific vs. genus specific vs. panfungal). Once suitable probes had been identified and synthesised they had to be shown to be specific to the target species and also to possess the required sensitivity to detect small amounts of target DNA in a 50µl reaction volume and finally in a blood sample.

Additionally, since the methodology was novel the project was an opportunity to investigate some of the issues which surround the mechanism behind

detection of target DNA. While pursuit of the primary aim of the project prevented a thorough examination of the issues in question, some of the more common or problematic issues that were encountered were explored to a degree and any findings of relevance were reported (see chapters 3 & 7).

1.6 Strategy

As discussed above the number of fungal pathogens implicated in human disease is large and the design of probes capable of species-level identification for all of them would be extremely difficult and exhibit diminishing returns on the effort that would have to be invested in such a task. Instead it would be sensible to have a smaller suite of probes that covered the most commonly occurring fungal pathogens as well as those most frequently implicated in antifungal resistance. The majority of infections are caused by five species of the genus *Candida* and two of these, *C. glabrata* and *C. krusei* are commonly associated with antifungal resistance. It was therefore decided that the assay should be developed to detect and identify these five species. Further to the design of the species-specific probes it was decided to try and design of a pan-fungal probe as well. This approach would allow the early detection of an invasive fungal infection caused by uncommon species and would be a valuable early warning which would allow empiric antifungal treatment to be administered while further tests can be performed on the patient with the knowledge that fungal involvement has been demonstrated.

The strategy of probe design that was therefore employed was to have a suite of 6 probes: 5 species specific probes targeted to *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* to detect the most common species of *Candida* implicated in invasive fungal infections, and a single pan-fungal probe that would detect DNA from any fungal species present in a sample. Fig 1.1 shows how the results of a multiplex reaction with the 5 + 1 probe suite could help inform treatment of a patient with suspected IFI. As mentioned before, no approach could cover all eventualities of diagnosing a fungal infection but this strategy represents a useful advance in identification of fungal pathogens that aids in guiding treatment.

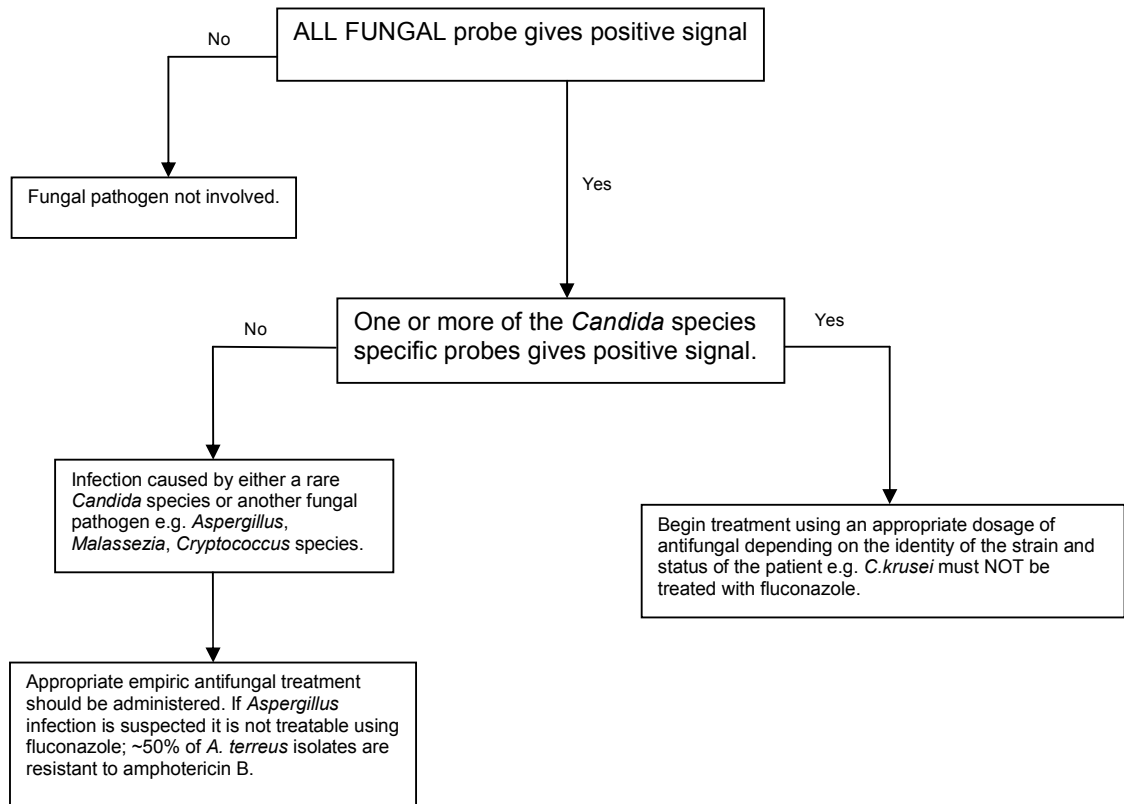


Figure 1.1 Decision tree which demonstrates how the 5 *Candida* species specific probes and the pan-fungal probe would enable clinicians to make a more informed decision of how to treat an invasive fungal infection.

The next question was the choice of DNA target for the probes. PCR-based assays can be developed to detect any target DNA for which PCR primers and a specific probe can be designed. Pathogenic organisms, including fungi, are particularly amenable in this respect as numerous DNA sequences from such organisms are available in public databases. Areas of particular interest are the internal transcribed spacer (ITS) regions of the rRNA cistrons (fig 1.2). The ITS regions have been targeted in a number of methodologies (Fujita *et al.*, 1995; Turenne *et al.*, 1999; Guiver *et al.* 2001; Leaw *et al.*, 2007; Lau *et al.*, 2008) and are very useful for design of species-specific primers and probes for identification as they are present in multiple copies between ~40 and ~150 copies per haploid genome in fungi (Ganley & Kobayashi, 2007), they exhibit considerable variability between species and are flanked by the highly conserved rRNA genes which facilitate the design of more universal

primers and probes (Iwen *et al.*, 2002). It was decided to target the ITS2 region for the design of the probes for species-specific identification and use fungal universal primers targeted to the flanking 5.8S and 26S rRNA genes to amplify the ITS2 region. For design of the panfungal primers and probe the rRNA genes were used and eventually the 18S rRNA gene was settled on as the most favourable target. The use of fungal universal primers has the disadvantage that any fungal species which contaminates the PCR mix will present a target for the primers. Provided the species-specific probes have been well designed the probability of a false positive result is low; however, the probability of a false negative result is increased as the contaminant DNA will act as a target for the generic primers and so compete with target DNA for amplification. This would be more likely to affect reactions where very low amounts of target DNA are present as the contaminant DNA will be able to sequester the primers and become the dominant amplicon early in the reaction. It would be possible to design species-specific primers, however this was not attempted as it would make development of the assay for multiplex detection more difficult, due to potential primer-primer interactions and would make addition of further species-specific probes to the suite more difficult to achieve.

Type strains of each of the five *Candida* species of interest were used to show that detection is possible using the appropriate probe while their specificity was tested experimentally against a panel of nine non-target *Candida* species that are implicated in human disease as well as against human DNA. The robustness of the probes was tested against wild isolates of the appropriate species and clinical isolates were used where possible. The robustness of the pan-fungal probe was tested experimentally against as wide a range of fungi as possible. The limit of detection (LOD) of the probes was ascertained firstly in assays with extracted DNA

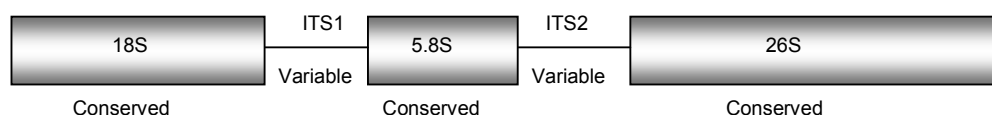


Figure 1.2. Schematic overview of the rRNA cistron showing the conserved rRNA genes flanking the variable ITS regions.

diluted to the level of 1 genome present in a 50µl PCR reaction. If this is achieved successfully the LOD of extracted DNA spiked into healthy blood samples will be investigated. Finally detection of small numbers of whole cells spiked into healthy blood samples will be attempted.

If the assay can achieve all of the above goals satisfactorily then it will have demonstrated the utility of Atlas Genetics' method and placed the fungal detection assay in good stead for further clinical testing and potential adaptation for use on the automated point of care system proposed by Atlas Genetics.

Chapter 2 - Materials and Methods.

2.1 Culture acquisition

Type strains of 10 *Candida* species were obtained from the Centraal Bureau voor Schimmelcultures (CBS) and wild strains were acquired from the culture collection at the University of Bath and from other donors – table 2.1 lists the strains that were used and includes information concerning their geographical origin and site of isolation, where available. All species used had their identity confirmed by sequencing of the ITS2 region. All yeasts were cultured on yeast-peptone-dextrose (YPD) agar (1% yeast extract (Oxoid), 2% Bacto peptone (Oxoid), 2% glucose (Sigma), 2% Bacto agar (Difco)) at 28°C for 48 hours.

Samples of type strain cultures were prepared for long term storage using the following method: a growing colony was picked from YPD agar using a sterile loop, transferred to 5ml liquid YPD and incubated overnight at 28°C with shaking. 1ml samples of each culture were then aliquoted into 2ml microcentrifuge tubes and 1ml of 20% glycerol added as a cryoprotectant before transfer to -20°C storage.

Filamentous fungi were cultured on potato dextrose agar (PDA) (Oxoid) and stored at 4°C until DNA extraction.

2.2 Steps to limit contamination

In order to prevent contamination the setup of PCR reactions and the processing of blood samples was performed in a safety cabinet or flow hood which was regularly disinfected with 70% ethanol and sterilized using ultra violet (UV) irradiation. The air flow remained switched off while the mix was prepared to avoid contamination of samples by airborne PCR amplicon. All stock reagents were aliquoted into working stock solutions to limit potential contamination and problems associated with freeze-thawing.

Table 2.1. List of fungal strains used. Type strains are designated with ^(T).

Species name	University of Bath code	Culture collection number	Origin	Species name	University of Bath code	Culture collection number	Origin
<i>C. albicans</i> ^(T)	LN1568	CBS 562	Uruguay, interdigital candidiasis in human	<i>C. krusei</i> ^(T)	LN1562	CBS 573	Sri Lanka, bronchomycosis, human sputum
<i>C. albicans</i>	LN2327	-	U.K., blood	<i>C. krusei</i>	LN1606	-	Uganda, human
<i>C. albicans</i>	LN1640	NCPF 3281	U.K., patient sample	<i>C. krusei</i>	LN1613	-	Uganda, human
<i>C. albicans</i>	LN4350	-	Endocarditis	<i>C. krusei</i>	LN1653	-	U.K. patient sample
<i>C. albicans</i>	LN3664	-	Human urine sample	<i>C. krusei</i>	LN1654	-	U.K. patient sample
<i>C. glabrata</i> ^(T)	LN1563	CBS 138	Human faeces	<i>C. lusitaniae</i> ^(T)	LN1601	CBS 4413	Portugal, caecum of pig
<i>C. glabrata</i>	LN2231	-	U.K., blood culture	<i>C. famata</i> ^(T)	LN1566	CBS 767	Carlsberg lab
<i>C. glabrata</i>	LN2230	-	U.K., blood culture	<i>C. kefir</i> ^(T)	LN1564	CBS 834	Netherlands, kefir grain
<i>C. glabrata</i>	LN2228	-	U.K., blood culture	<i>C. dubliniensis</i> ^(T)	LN1565	CBS 7987	Ireland, oral candidiasis in human
<i>C. glabrata</i>	LN2244	-	U.K., blood culture	<i>C. guilliermondii</i> ^(T)	LN1602	CBS 566	Bronchomycosis, human
<i>C. parapsilosis</i> ^(T)	LN1567	CBS 604	Puerto Rico, case of sprue in human	<i>C. zeylanoides</i>	LN1666	NCPF 8426	Human
<i>C. parapsilosis</i>	LN2450	-	Turkey	<i>C. pelliculosa</i>	LN1662	-	Human
<i>C. parapsilosis</i>	LN2451	-	U.K.	<i>S. cerevisiae</i>	LN2208	-	High vaginal swab
<i>C. parapsilosis</i>	LN2235	-	U.K., blood culture	<i>P. fermentans</i>	LN2308	-	-
<i>C. parapsilosis</i>	LN2462	-	U.K., blood culture	<i>C. inconspicua</i>	LN1649	NCPF 8625	Sputum, human
<i>C. tropicalis</i> ^(T)	LN1569	CBS 94	Bronchomycosis patient, human	<i>C. lipolytica</i>	LN1655	NCPF 8630	Human
<i>C. tropicalis</i>	LN3133	-	Inclusions in vesicles	<i>Cr. gatii</i> *	N/A	-	Meningo-encephalitic lesion
<i>C. tropicalis</i>	LN1608	-	Uganda, human	<i>Cr. victoriae</i>	LN3422	-	-
<i>C. tropicalis</i>	LN1611	-	Uganda, human	<i>A. nidulans</i>	LN2325	-	-
<i>C. tropicalis</i>	LN1614	-	Uganda, human	<i>F. oxysporum</i>	LN1107	-	U.S.A.

* *Cr. gatii* extracted DNA was used rather than cultures.

CBS: Centraal Bureau voor Schimmelcultures, Netherlands.

NCPF: National Collection of Pathogenic Fungi, U.K.

2.3 Whole blood acquisition and treatment

Blood from a healthy donor was collected by a trained individual into 6ml vacutainer tubes. For samples which were to be kept as whole blood or be processed to yield plasma, K₂EDTA-coated vacutainers (BD Biosciences) were used to prevent clotting and upon collection of blood the vacutainer was inverted 8 – 10 times to ensure adequate mixing of the anti-coagulant with blood. For samples which were to be processed to yield serum, untreated vacutainers were used for collection and the blood was incubated at 28°C for 1 hour to allow clot formation. The vacutainer was then centrifuged at 1400 relative centrifugal force (rcf) for 10mins at 4°C. 1ml aliquots of the serum fraction were then pipetted into 2ml sterile cryovials. For preparation of plasma samples the anti-coagulated blood sample was centrifuged at 1400rcf for 10mins at 4°C and then 1ml aliquots of the plasma fraction were aliquoted into 2ml sterile cryovials. All of the plasma, serum and whole blood samples were stored at -80°C until used.

2.4 DNA extraction

2.4.1 Extraction from liquid cultures

The QIAgen DNeasy extraction kit was used for all extractions and the manufacturer's protocol was followed with some modifications that were appropriate for treatment of fungal material. The yeast cell count in a stock cell suspension was estimated by spectrometry according to the method described in Amberg *et al.* (2005) and a solution of approximately 5×10^6 yeast cells was prepared by appropriate dilution of the stock and centrifuged at 10,000 rpm for 10mins to pellet the cells. The supernatant was removed and the cell pellet resuspended in 500ul of a lyticase lysis buffer which comprised 10U ml⁻¹ lyticase from *Arthrobacter luteus* (Sigma), 50mM Tris, pH 7.5, 10mM EDTA and 28mM β-mercaptoethanol; the lyticase digestion was performed at 37°C for 30mins. For filamentous fungi, the PDA was flooded with 1ml of a sterile solution of 0.9% saline and agitated to transfer fungal material to the solution. The saline was then collected, centrifuged, resuspended in lyticase buffer and incubated as above. Following treatment by lyticase the sphaeroplasts were collected by centrifugation at 10,000rpm for 10mins, the supernatant removed and the sphaeroplasts resuspended in 180 µl buffer

ATL to which 20µl of a proteinase K solution provided in the kit was added and the mixture vortexed and incubated for 45mins at 55°C. After proteinase treatment, 200µl of buffer AL and 200µl of absolute ethanol were added to the samples, mixed thoroughly and the samples left on ice for 30mins to increase precipitation of DNA and enhance final yield. The samples were then placed in silica-based spin columns and wash steps with 500µl of buffer AW1 and AW2 were applied as per the manufacturer's instructions. DNA was then eluted twice from each column using 50µl of the elution buffer for each elution and a single 1.5ml tube was used to collect both eluates. The concentration of eluted DNA was estimated by spectrometry. All DNA was stored at -20°C.

2.4.2 Extraction from blood samples spiked with *C. albicans* genomic DNA.

The required number of 1ml blood samples (whole blood or plasma) were removed from storage at -80°C and allowed to thaw. *C. albicans* DNA was removed from -20°C storage and allowed to thaw. A 5ml sterile bijoux tube had the required amount of *C. albicans* genomic DNA added and 1ml of the blood sample was then added to the bijoux. The solution was agitated gently to allow mixing of the DNA in the blood. Extraction of the *C. albicans* DNA from blood was performed using GenElute Bacterial Genomic DNA extraction kit (Sigma) with considerable modification.

The spiked blood samples had 2ml of a proprietary lysis buffer added and were incubated for 5mins at room temperature with occasional gentle agitation to allow mixing and complete lysis of blood cells. Meanwhile the required number of extraction columns was prepared using Column Preparation Solution as per manufacturer's instructions. After the 5min incubation of blood samples 700µl of the lysate was applied to the spin column and centrifuged at 12,000g for 1min to allow DNA to bind to the column. The flow-through was discarded. The above step was repeated until all of the lysed blood sampled had been spun through the column. After the final centrifugation the collection tube was discarded and replaced with a fresh one. 500µl of Wash Solution 1 was applied to the column and centrifuged at 12,000g for 1min. The collection tube was discarded and replaced. 500µl of

the wash solution with added ethanol was applied to the column and centrifuged at 12,000g for 1min. This time the flow through was discarded, the column placed back in the same collection tube and centrifuged for a further 1min. The collection tube was then discarded and replaced with a fresh one. Elution of DNA was initially performed by adding 100µl of elution buffer to the column, incubating at room temperature for 30s and centrifuging at 12,000g for 1min. 30µl of this eluate was used as template in PCR reactions. However, to improve the yield when very small amounts of *C. albicans* DNA were spiked into blood the elution buffer was prewarmed to 60°C and 34.25µl was applied to the spin column, incubated for 30s at room temperature and centrifuged at 12,000g for 1min. The eluate was then reapplied to the spin column, incubated and centrifuged for a second time. This entire eluate was then used as template in PCR amplifications.

2.4.3 Extraction from blood samples spiked with *C. albicans* cells.

To obtain cell suspensions of the appropriate number of cells to spike into blood samples, growing colonies of *C. albicans* were picked with a sterile loop and resuspended in 500µl sterile H₂O. This stock solution was diluted 10-fold and 50-fold and cell-density estimates of the three solutions were made using an improved Neubauer counting chamber. After confirming that the estimates were in good agreement with one another, the cells were further diluted to yield solutions of 1cell µl⁻¹ and 0.1cell µl⁻¹. The cell densities were confirmed retrospectively by performing plate counts.

The extraction procedure was very similar to the method outlined in section 2.4.2 but differed in the pretreatment step. After thawing the blood samples, 10µl of the appropriate *C. albicans* cell suspension was added to a 7ml sterile bijoux and then the 1ml blood sample was added and the solution mixed. 250µl of a lysis buffer containing 200U ml⁻¹ lyticase (see section 2.4.1) was added and the reaction mix incubated at room temperature for 5min with gentle agitation. After this step the extraction continued with the addition of 1ml of the proprietary lysis buffer and was identical to the procedure outlined in section 2.4.2. Note that 34.25µl of prewarmed elution buffer was always used for elution of DNA extracted from *C. albicans* cells.

2.5 Primers and probes

Fungal universal primers ITS3 and ITS4 (White *et al.*, 1990) were obtained from Sigma. The primers amplified a region between the 3' end of the 5.8S rRNA gene and the 5' end of the 28S rRNA gene, which encompassed the ITS2 region (see fig 2.1). Sequences of species-specific probes for detection of five *Candida* species that targeted this region were obtained from Shin *et al.* (1999). These species-specific sequences detected the following species (the name in parentheses was the name given the electrochemically labelled probe used in this work): *C. albicans* (CA PR), *C. tropicalis* (CT PR), *C. parapsilosis* species-complex (CP PR), *C. glabrata* (CG PR) and *C. krusei* (CK PR). The sequences were used to synthesize oligonucleotides which were labeled at the 5' terminal nucleotide with a proprietary ferrocene compound by ATDBio (Southampton). Some of the original probes produced unsatisfactory results and a number of different probes were designed. Furthermore, the ITS3 and ITS4 probes were not suitable for satisfactory amplification of low amounts of target DNA. Several new forward and reverse primers were designed by eye using a matrix of fungal ITS2 sequences. Following tests of all primer combinations experimentally the primer pair ITS3.3 and ITS4.2 was found to yield the best results and these primers were used for amplification of low amounts of fungal DNA.

A pan-fungal probe (PanF PR) was designed manually by analyzing a matrix of fungal 18S ribosomal DNA sequences that had been obtained from GenBank and identifying an appropriate target site for a probe (see below). This probe was then BLASTed (Altschul *et al.*, 1997) against a database containing human, trypanosome, plasmodium and fungal 18S ribosomal DNA sequences to ensure that it would hybridize to the fungal sequences but not to human or other organisms' DNA. The same matrix was then used to identify potential primers to amplify the area surrounding the PanF PR target site. Following PCR trials the forward primer Fungal18sF2 and the reverse primer Fungal18sR4 were chosen for use with the PanF PR probe. All of the primer and probe sequences used are listed in table 2.2. Primers were synthesized by either Sigma or IDT Technologies.

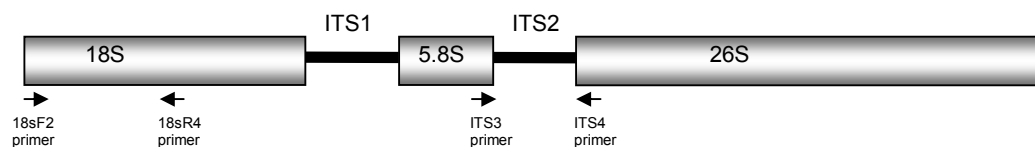


Figure 2.1. Schematic representation of the 18S, 5.8S and 26S rRNA genes with the internal transcribed spacer (ITS) regions. The target sites of the primers have also been indicated.

2.6 Assessing cross-reactivity of probes using bioinformatics.

Fungal ITS2 sequences were obtained from Genbank and sequence alignments performed using the MEGA Version 4.0 software (Tamura *et al.*, 2007). This alignment was used as a database for a BLAST search with each of the *Candida* species-specific probes. Any non-target species that were found to have $\geq 60\%$ identity to any of the species-specific probes were used in assays to test for cross-reactivity.

A sequence alignment of 18s rRNA genes from fungi but which also included human, plasmodium and trypanosome sequences was constructed and used to identify primers and probes for universal detection of fungi. Potential primers were screened experimentally to find the best combination, but to decide between potential probe target sites a BLAST search was performed using the 18s rRNA alignment as the database against which the potential probe sequences were screened. In this case it was desirable that all of the fungi had a high percentage of matched bases while the non-fungal sequences were as low as possible.

Table 2.2. Primer and internal probe sequences used in the electrochemical detection assay. All probes had a diferrocene label attached by a carbon linker molecule to the 5' terminal nucleotide.

Forward Primer Name	Primer sequence (5' - 3')	Reverse Primer Name	Primer sequence (5' - 3')
ITS3	GCA TCG ATG AAG AAC GCA GC	ITS4	TCC TCC GCT TAT TGA TAT GC
ITS3.1	GGA TCT CTT GGT TCT CGC AT	ITS4.1	CCT ACC TGA TTT GAG GTC A
ITS3.2	CAT CGA ATC TTT GAA CGC ACA	ITS4.2	AGT CCT ACC TGA TTT GAG G
ITS3.3	TGC CTG TTT GAG CGT CAT TTC	ITS4.3	CCT ACC TGA TTT GAG GTC
Fungal18sF2	GCC ATG CAT GTC TAA GTA TAA	Fungal18sR4	CCT CGT TAA GGG ATT TAA ATT GT
Probe Name	Probe Sequence (5' - 3')	Species detected	
CA PR	ATT GCT TGC GGC GGT AAC GTC C	<i>C. albicans</i>	
CA PR2	TGC GGC GGT AAC GTC CAC CA		
CA PR3	ATC GCT TTG ACA ATG GCT TA		
CT PR	CAA AAC GCT TAT TTT GCT AGT GGC C	<i>C. tropicalis</i>	
CP PR	GGT ACA AAC TCC AAA ACT TCT TCC A	<i>C. parapsilosis</i>	
CG PR	TAG GTT TTA CCA ACT CGG TGT TGA T	<i>C. glabrata</i>	
CG PR2	CCT CGT GTC CCA CAT ACT GA		
CG PR3	TCA GTA TGT GGG ACA CGA GCG CAA G		
CK PR	AGT GGC CCG AGC GAA CTA GAC TTT T	<i>C. krusei</i>	
PanF PR	GGT GAT TCA TAA TAA CTT TTC G	All fungi	

2.7 PCR conditions

2.7.1 PCR conditions for testing assay specificity

All PCR reactions were performed in Peltier PTC thermal cyclers (MJ Research). Asymmetric PCR conditions using a 5:1 ratio of reverse:forward primers were used to generate an excess of single stranded target DNA for the assay. PCR reactions to amplify target for use in electrochemical assays were set up in at least triplicate. PCR reactions were carried out using GE Healthcare's Illustra *Taq* DNA polymerase in a volume of 30µl containing the following reagents: PCR buffer (10mM Tris-HCl, pH 9.0, 50mM KCl, 1.5mM MgCl₂) (GE Healthcare), 100nM forward primer, 500nM reverse primer, 0.15mM each dNTP, 1U IllustraTaq DNA polymerase (GE Healthcare) and 2µl extracted DNA, corresponding to 10-100ng. The remaining volume was made up to 30µl with molecular grade water (Sigma; 0.1µm filtered, DNase, RNase and protease free). These conditions were satisfactory for the *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* specific probes but not the *C. albicans* specific probe. Establishing an assay for detection of *C. albicans* DNA proved difficult and required alteration of some of the PCR components - an alternative, in-house buffer was used to amplify *C. albicans* DNA that contained 10mM Tris-HCl (pH 8.3), and 50mM KCl while MgCl₂ was added separately at a concentration of 3mM. For all of the probes the amplification conditions used for amplification of the ITS primer pairs were: 94°C for 1min; 94°C for 20s plus 55°C for 20s plus 72°C for 20s for 40 cycles; 72°C for 3mins. For PCR amplification using the 18s primers the above program was altered to have an extension step of 72°C for 40s as the product was longer.

2.7.2 PCR for DNA sequencing

PCR reactions to amplify target DNA for sequencing were carried out using the 'standard' PCR conditions described above but in a reaction volume of 50µl and symmetric PCR conditions were used. Following PCR the products were cleaned using the Qiaquick PCR cleanup kit (Qiagen) following manufacturer's instructions and DNA concentration was estimated using a low molecular weight mass ladder (Fermentas). Sequencing reactions were carried out by MWG or Cogenics. BLAST searches were performed against

the GenBank sequence database to confirm the identity of the sequenced fungal isolates.

2.7.3 PCR conditions for testing assay sensitivity

The PCR conditions described above were inadequate for amplification of low amounts of target DNA and had to be changed. Research into alternative *Taq* DNA polymerases and PCR buffers by researchers at Atlas Genetics had identified a better enzyme and improved buffer solution. My conditions were therefore changed to use the the hotstart *Taq* polymerase Jumpstart (Sigma) and a 10X PCR buffer comprising 100mM Tris-HCl (pH 8.3), and 500mM KCl which was made in the Atlas Genetics laboratory. The PCR used a total volume of 50 μ l containing the following reagents: PCR buffer (see above), 100nM forward primer, 500nM reverse primer, 0.15mM each dNTP, 3mM MgCl₂ and 2.5U Jumpstart *Taq* DNA polymerase (Sigma) made up to 50 μ l using molecular biology grade water (Sigma). The cycling conditions used were: 95°C for 1min; 94°C for 30s plus 58°C for 30s plus 72°C for 1min for 40 cycles; 72°C for 3mins.

2.7.4 Visualisation of PCR products

A 10 μ l aliquot of the PCR products was added to 2l of 6X loading dye and visualized on a 1% (w/v) agarose gel made up in Tris-Borate-EDTA (TBE) buffer (0.09M Tris base, 0.09M boric acid, 2mM EDTA pH 8.0) containing 0.5 μ g ml⁻¹ ethidium bromide to confirm the presence of amplified target DNA. Samples were run at 10V cm⁻¹. 20 μ l of the remaining PCR product was used for the electrochemical assay.

2.8 Measurement of Electrochemistry

A probe mix was prepared for each sample to be assayed containing the following reagents: 3 μ M of the relevant probe and 10U T7 exonuclease (New England Biolabs) made up to 5 μ l with molecular grade water (Sigma). This probe mix was added to 20 μ l of PCR product and incubated for 20mins at 37°C to allow hybridization of the probe to single stranded target DNA and subsequent digestion of the duplex by T7 exonuclease. Once incubation was complete 20 μ l of the probe reaction mix was pipetted onto screen printed

carbon electrodes with a silver/silver-chloride reference electrode. The electrodes used for specificity testing were manufactured by Gwent Electronic Materials Ltd. and those used for sensitivity testing were manufactured by G. M. Nameplate. The observed current due to oxidation of the released ferrocene-labeled nucleotide following DPV was measured using a potentiostat (Autolab PGSTAT30, EcoChemie) and dedicated software (GPES version 4.9, EcoChemie). DPV measurements were made using the following settings: modulation time 0.04s; interval time 0.1s; initial procedure -0.1V; end potential 0.5V; step potential 0.003V; modulation amplitude 0.05V.

2.8.1 Synthetic target assay

5 μ M of a 60-mer synthetic oligonucleotide molecule (Sigma) which matched 60bp of the ITS2 region of the target species, and contained the probe binding site, was added to 2 μ l of 10X Illustra Taq PCR buffer. 3 μ M of the appropriate probe and 10U of T7 exonuclease were added, followed by H₂O to give a final volume of 20 μ l. Reaction mixes were incubated at 37°C for 20mins as above and the entire 20 μ l sample used to obtain electrochemistry readings as above.

Chapter 3 - Model of the Electrochemical Assay.

3.1 Introduction.

The assay under development by Atlas Genetics is novel, but operates in a similar way to other well established molecular sensing techniques. Perhaps the most closely related method is the Taqman assay using a dual-labelled oligonucleotide probe. This procedure is a modification of conventional PCR which allows amplification to be monitored by the inclusion in the reaction of a third DNA oligonucleotide which is labelled at the 5' end with a molecule capable of fluorescing when excited by U.V. light and at the 3' end by a molecule which quenches the fluorescence of the first when they are in close proximity. This dual-labelled oligonucleotide binds to a target site within the area being amplified by the primers during the annealing step of PCR and is then digested by the 5' - 3' exonuclease activity of *Taq* polymerase. The fluorescence-labelled mononucleotide is then able to diffuse away from the quencher-labelled mononucleotide and this produces a measurable increase in fluorescence when U.V. light of the appropriate wavelength is applied to the sample. As amplification continues the amount of available target increases and so the amount of fluorescence increases. There is always a background fluorescence associated with these assays, caused by weak fluorescence of the quenched label on the 5' end of the intact probe; it provides a base level of fluorescence which is essential for determining when DNA amplification reaches the exponential phase and is usually used quantitatively to determine the amount of template DNA present at the start of the reaction.

The Atlas Genetics electrochemical assay also uses a third DNA oligonucleotide for detection of amplified products but this molecule is labelled at only its 5' end with a proprietary ferrocene-based molecule which can be oxidised, producing a measurable electric current, when voltage of a particular value is applied. This latter property can be exploited to allow multiplex detection of probes labelled with ferrocene molecules that oxidise at different voltages, in a similar way to Taqman assays that use different fluorescent probes that emit light at different wavelengths following excitation. The assay

differs further from the Taqman method in that it is an end-point detection assay - amplification of target by PCR is first completed and then the probe is added to the reaction alongside a dsDNA-specific exonuclease. The probe hybridises to amplified target and is digested to release mononucleotides. The ferrocene-labelled mononucleotide products of this digestion diffuse to the surface of an electrode where they are oxidised following application of voltage sweep and it is the current produced by the transfer of electrons during this event that is recorded (Fig 3.1). A background signal is also associated with the electrochemical assay due to oxidation of low levels of undigested probe present at the electrode surface. Reaction conditions can have an effect on the background current and can result in either an increase or a decrease in the signal.

While a model of how the assay produces current has been suggested, exactly how the different factors involved in the assay affect current output has not yet been precisely established. Some of these factors will now be introduced and data acquired during my research that sheds light on aspects of them will be discussed. It is important to note that the results which follow were derived at different times during the project and some were performed under different conditions than others. However, at each point that a change in procedure took place comparative experiments were performed to ensure that the new system gave similar, comparable results to the old system. The most notable improvement was the eventual reduction of background current to zero which can be seen in some of the results. The possible reasons for this are discussed in section 3.5 below.

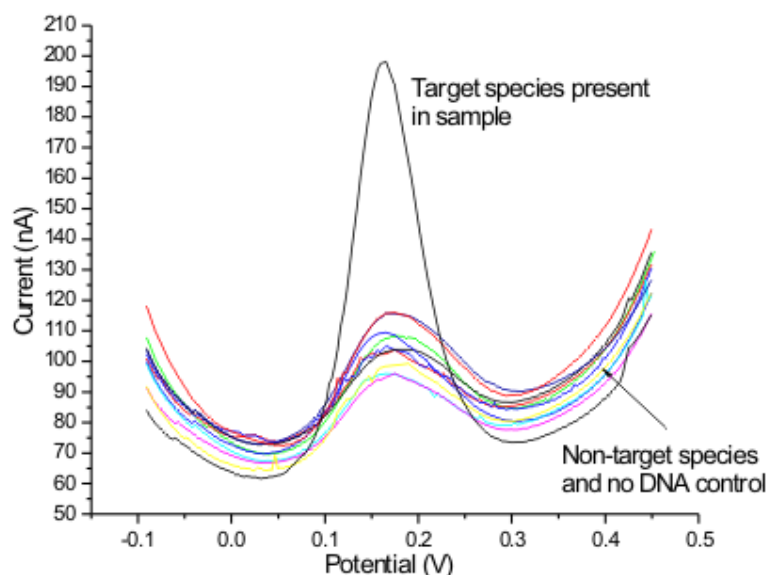


Figure 3.1. Example data showing current peaks produced following oxidation of ferrocene in the assay. A voltage sweep from -100mV to +500mV was used and peak oxidation occurred at ~180mV. The probe used was CT PR.

3.2 PCR amplification.

3.2.1 Asymmetric PCR

The first part of the assay, PCR, is of critical importance as the current generated by the assay is proportional to the amount of target DNA present which is in turn dependent on PCR amplification; the procedure must be optimal to ensure the production of as much target as possible. Asymmetric PCR conditions using a 5 to 1 ratio of target strand synthesising primer to complementary strand synthesising primer (in this assay a 5:1 ratio of ITS4:ITS3) for asymmetric PCR had previously been shown to yield optimal results for assays with bacterial target DNA (S. Boundy, pers comm) and it was decided to maintain these conditions for use in the fungal assay. One drawback to asymmetric PCR compared with symmetric PCR is that the exponential phase of the reaction does not last as long, hence less product is formed in the final cycles of the reaction. However, symmetric PCR produces a population of predominantly dsDNA molecules which would mean that the target DNA molecules would be duplexed with their complementary strands making it difficult for the probes to bind. This would result in lower currents being produced compared to asymmetric PCR conditions which, while not

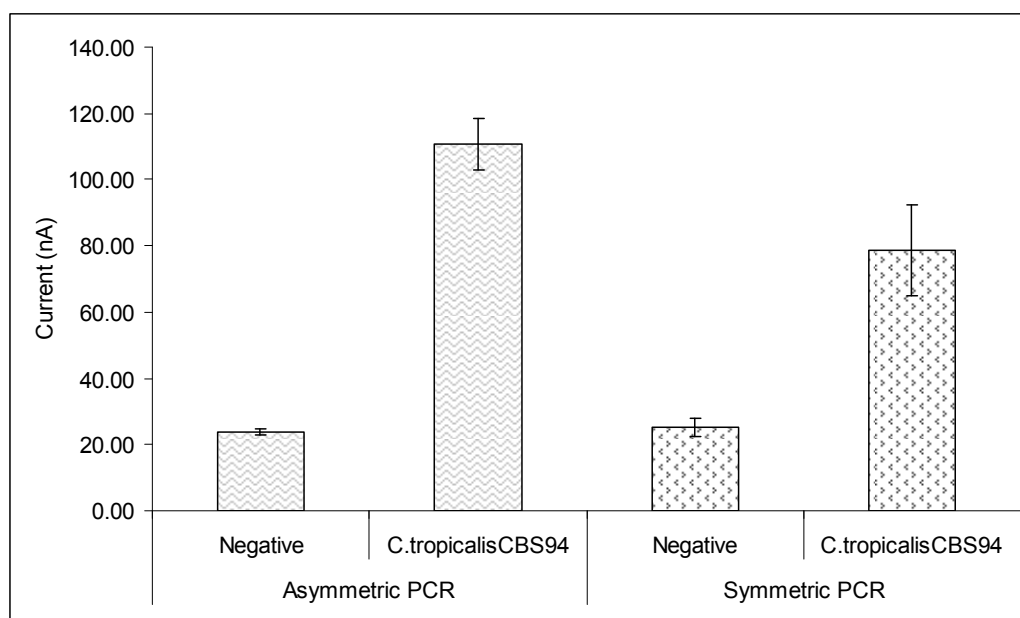


Figure 3.2. Comparison of the effect of asymmetric and symmetric PCR conditions on the current output of the assay using target derived from *C. tropicalis* DNA and the probe CT PR. Error bars represent SD (n=4).

producing as much target DNA, produce more ssDNA. Increased ssDNA target was hypothesised to yield a better signal compared to symmetric PCR conditions. The two methods were directly compared using the same template and probe and the mean results are displayed in Fig. 3.2. The mean current generated with amplicon derived from asymmetric conditions was significantly higher than the mean current generated by symmetric conditions: $110.6 \pm 7.9\text{nA}$ compared with $78.7 \pm 13.9\text{nA}$ ($P < 0.01$). Despite the statistical significance, the difference between these mean values was only 31.9nA which was a relatively small difference and one that could occur between measurements of replicates with the same experimental conditions.

The relatively high currents generated by the assay in the presence of dsDNA target suggested that some mechanism was allowing the probe to hybridise to the target and be digested by T7 exonuclease with reasonable success. One possibility was that the probe molecule was able to displace the complementary strand from the target site and hybridise in a similar manner to single strand invasion events that occur during homologous recombination. Another possibility was that the ITS4 primer, which synthesised the probe's

target strand, was a more efficient primer than ITS3 and so, even when the primers were present at equal concentrations, the products of the reaction still contained a proportion of ssDNA target which produced a signal when analysed electrochemically. The design flaws associated with primer ITS3, which may have limited its efficiency, are discussed further in section 3.2.2.

The exact reasons for production of a relatively high signal by symmetric PCR were not investigated further. The key conclusion was that asymmetric PCR conditions gave better results in the electrochemical assay and were incorporated into the routine assay procedure.

3.2.2 Primer Design

The efficiency of primers used for the PCR was also absolutely critical in obtaining a good signal output, especially when a low amount of template DNA was being used. The initial primer pair that was used, ITS3 and ITS4, had produced good results when target fungal DNA was present in large amounts in PCR i.e. tens of nanograms. However, when the amount of DNA present in the reaction was reduced to femtogram levels detection was no longer possible, either visually by running products on a gel, or electrochemically. Upon examination of the primers it was discovered that two of the 3' terminal nucleotides of each primer were complementary to both itself and its partner (Fig 3.3A and B) and it was thought that this potential self-dimerising behaviour might have affected the amplification of target DNA, particularly when low amounts of target were present because, when little template DNA is present, mispriming in the early stages of PCR has a much more pronounced effect; at best it may sequester primer molecules and prevent them from binding to the template genomic DNA, and at worst may actually produce a primer-dimer product which becomes the preferred template in the early stages of the reaction. In either case, amplification of the desired target is reduced with the effect that the signal produced in the assay is also reduced. New primer pairs were therefore redesigned to other conserved regions within the 5.8s and 28s rRNA.

ITS4: 5'-TCCTCCGCTTATTGATATGC-3'	
ITS3: 3'-CGACGCAAGAAGTAGCTACG-5'	

Figure 3.3A. Potential hetero-dimerisation between primers ITS3 and ITS4.

ITS3: 5'-GCATCGATGAAGAACGCAGC-3'	
ITS3: 3'-CGACGCAAGAAGTAGCTACG-5'	

ITS3: 5'-GCATCGATGAAGAACGCAGC-3'	
ITS3: 3'-CGACGCAAGAAGTAGCTACG-5'	

Figure 3.3B. Two potential self-dimerisation configurations with primer ITS3.

Note that ITS4 is also capable of self-dimerisation between its 3' termini.

Assays using these primers with low amounts of target DNA produced considerably higher values of current than those performed with the ITS3 and ITS4 primers; Figure 3.4 shows comparative results generated by each primer pair. The old primer pair, ITS3 and ITS4, gave a mean current of $72.4 \pm 2.4\text{nA}$ compared with $165.3 \pm 12.4\text{nA}$ and $83.8 \pm 6.6\text{nA}$ for primer pairs for ITS3.1/ITS4.1 and ITS3.2/ITS4.2 respectively. The considerably larger currents produced by assays utilising ITS3.1/ITS4.1 were a remarkable demonstration that differences in primer sequences, and therefore the efficiency of PCR, can produce large differences in current. Additionally, the DNA-free samples produced very similar values of current between the primer pairs, ranging between $12.8 \pm 4.4\text{nA}$ and $15.4 \pm 1.3\text{nA}$, indicating that the background current was independent of the PCR primers.

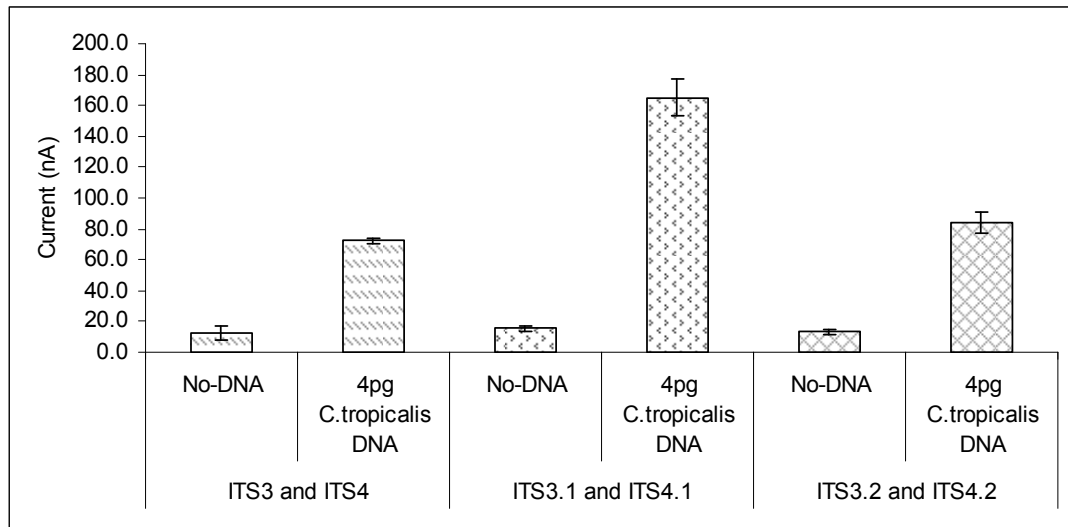


Fig 3.4. Mean currents produced in assays with the primer pair ITS3/ITS4 compared to the newly designed primer pairs ITS3.1/ITS4.1 and ITS3.2/ITS4.2. Error bars represent SD (n=3).

As mentioned previously, the primer pair ITS3/ITS4 was good enough to demonstrate the specificity of the species specific probes in assays that used large amounts of target DNA. Primer redesign had to be carried out to develop more efficient primers for amplification and detection of low amounts of target DNA. This is discussed further in section 5.2.2.

3.2.3 Magnesium Chloride (MgCl_2) Concentration.

The concentration of magnesium chloride (MgCl_2) is an important component of the reaction, both for efficient amplification of DNA by PCR and digestion of the bound probe:target heteroduplex by T7 exonuclease (Kerr & Sadowski, 1972). The initial experiment to determine the optimum concentration of MgCl_2 to add to the PCR used concentrations of 1.5mM, 2.0mM, 2.5mM and 3.0mM with DNA extracted from two strains of *C. albicans* and amplified by Illustra *Taq* polymerase (GE Healthcare). The gel image of the products is presented in Fig 3.5 and showed that only a MgCl_2 concentration of 1.5mM gave a product. As a result, the use of this concentration became incorporated into the assay procedure.

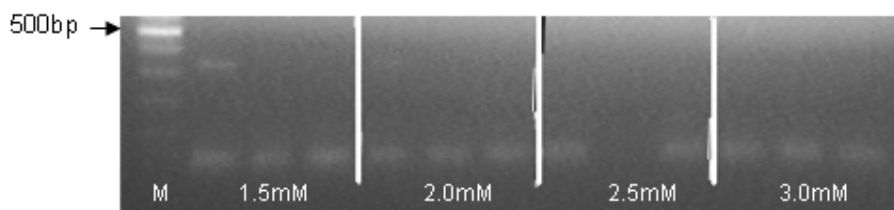


Figure 3.5. Results from the experiment to obtain the optimum concentration of MgCl_2 to add to the PCR. Each set of three samples is comprised of two reactions with DNA from 2 strains of *C. albicans* and the third sample in each set is a DNA-free negative reaction.

With hindsight, the above result was in all likelihood an anomaly, most likely due to use of poor quality extracted DNA; it was certainly odd that only one of the two *C. albicans* strains produced an amplicon at 1.5mM MgCl_2 . When 10 μl the extracted DNA was loaded onto a gel there was insufficient DNA present to be visualised, indicating a problem with DNA extraction. At the time, however, there were no other results from DNA extractions or from PCR amplifications available for comparison and so the assay procedure was set at a final concentration of 1.5mM MgCl_2 .

The opportunity to review the concentration of MgCl_2 used in the assay came when trying to improve the signal produced with the CA PR3 probe. Researchers at Atlas Genetics had optimised their assays and were regularly incorporating 3mM MgCl_2 into their reaction mixes; they had also switched to a different *Taq* polymerase. As these conditions were producing much better results with electrochemical assays it was decided to change to the new DNA polymerase and reconfigure the assay conditions for the fungal probe suite.

The MgCl_2 concentration was varied alone in the initial experiments (i.e. the Illustra *Taq* DNA polymerase was still used) and figure 3.6 shows the results of a MgCl_2 titration experiment. The results clearly showed that as the amount of MgCl_2 was increased in the experiment so too did the current

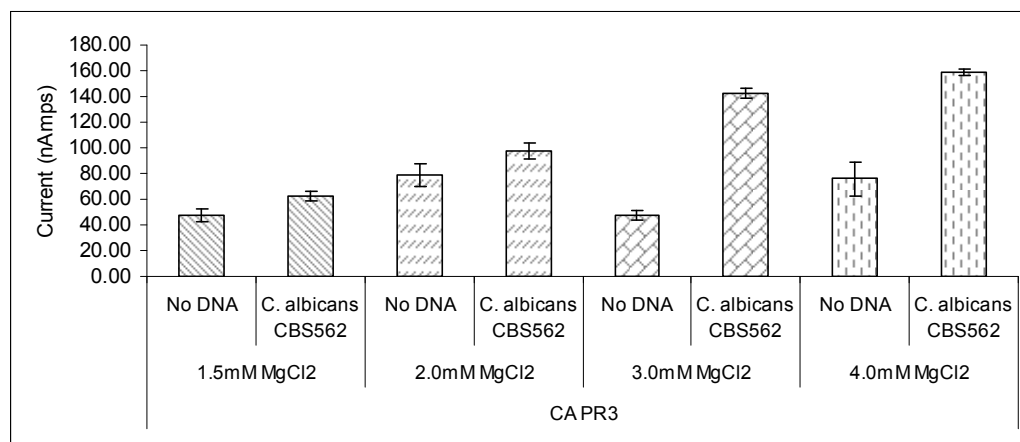


Figure 3.6. Mean currents from assays testing different concentrations of MgCl₂ added to the PCR reactions using Illustra Taq DNA polymerase. Error bars represent SD (n=3).

produced when target DNA was present and the mean currents ranged between $62.1 \pm 3.6\text{nA}$ at 1.5mM MgCl₂ and $158.3 \pm 2.6\text{nA}$ at 4mM MgCl₂. The currents produced by the DNA-free samples did not behave in the same way, however. There was an initial increase from $47.3 \pm 4.7\text{nA}$ to $78.6 \pm 8.6\text{nA}$ when the MgCl₂ concentration was increased from 1.5mM to 2mM and this $\sim 30\text{nA}$ increase was similar to the increase observed in the corresponding target-containing samples. However, when the MgCl₂ concentration was increased from 2.0mM to 3.0mM the mean background current actually *decreased* to $47.9 \pm 3.8\text{nA}$ while the target-containing sample increased by $\sim 46\text{nA}$ from $96.9 \pm 6.3\text{nA}$ to $142.4 \pm 3.4\text{nA}$. When the MgCl₂ concentration was increased to 4.0mM the background signal was again increased by $\sim 30\text{nA}$ to $75.7 \pm 13.4\text{nA}$ and the target-containing sample increased by only $\sim 16\text{nA}$ to $158.3 \pm 2.6\text{nA}$. The reason for the variation observed in the DNA-free samples was unclear and, in fact, only the result produced by the samples containing 3mM MgCl₂ was anomalous, the others gradually increasing as MgCl₂ concentration increased. Such an increase might be explained by a combined effect of both increased activity of T7 exonuclease as the MgCl₂ concentration was increased to the optimum concentration of 5mM suggested by Kerr and Sadowski (1972), as well as the increased formation of weakly bound primer:probe heteroduplexes. Fig 3.7 shows potential primer:probe interactions which, if formed during the assay, would be suitable targets for digestion and subsequent release of a ferrocene-labelled

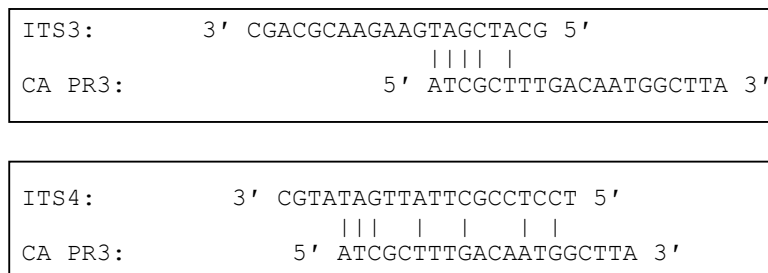


Figure 3.7. Possible primer:probe duplexes with probe CA PR3 and primers ITS3 and ITS4.

mononucleotide by T7 exonuclease. Such interactions would be more likely to occur in conditions of reduced stringency which would result from increasing the amount of $MgCl_2$ in PCR.

Ultimately, the reasons for the behaviour of the background current are unknown and were not investigated any further. A concentration of 3mM $MgCl_2$ was incorporated into the remaining assays to be carried out with Illustra *Taq* DNA polymerase and CA PR3 as this gave the best signal:background ratio and meant that the fungal assay conditions were more similar to the conditions used routinely by researchers at Atlas Genetics.

The assay also had to be optimised for use with the Jumpstart *Taq* DNA polymerase and another $MgCl_2$ titration experiment was performed to find the optimum concentration to work alongside this enzyme. A range of $MgCl_2$ concentrations of 1.5mM, 2mM, 3mM and 4mM were used in PCR with Jumpstart *Taq* polymerase and target DNA detected with CA PR3. The mean currents obtained are shown in fig 6.8 and were $19.7 \pm 2.5nA$, $57.5 \pm 4.1nA$, $119.7 \pm 13.6nA$ and $145.3 \pm 12.9nA$ for 1.5mM, 2mM, 3mM and 4mM respectively. This again demonstrated the general trend that increasing $MgCl_2$ led to an increase in current.

Since $MgCl_2$ is important for the T7 exonuclease stage of the assay as well as PCR, and the optimal $MgCl_2$ concentration for this enzyme has been reported to be 5mM (Kerr & Sadowski, 1972), the possibility that simply adding additional $MgCl_2$ to assay samples post-PCR to increase the $MgCl_2$

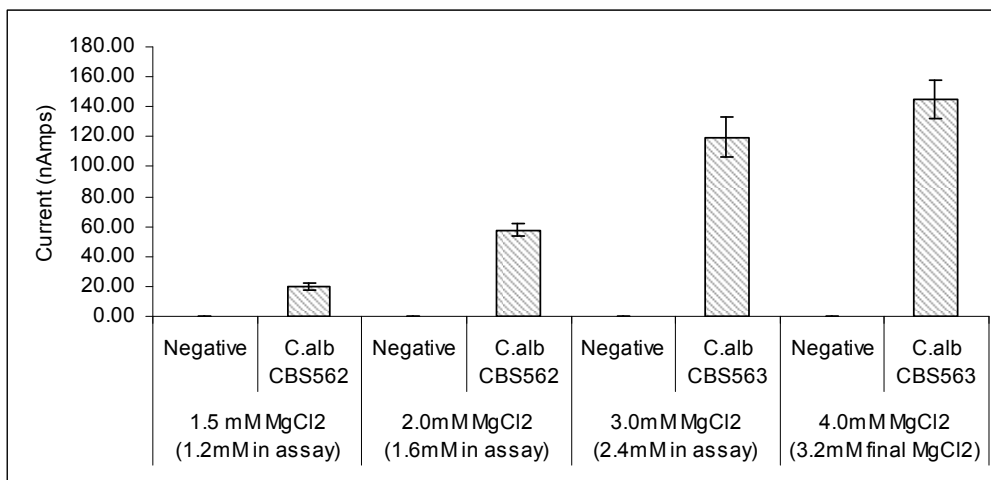


Figure 3.8. Mean currents produced by assays using Jumpstart *Taq* and different concentrations of MgCl₂ present in the PCR mix. The concentrations in parentheses indicate the final concentration of MgCl₂ in the assay following addition of 5 μ l probe-T7 mix to 20 μ l PCR product. The error bars represent standard deviation from the mean (n=3).

concentration to the optimum amount for T7 activity was investigated. Since the assay conditions when using Jumpstart DNA polymerase required a 50 μ l reaction it was possible to carry out a direct comparison using the remaining PCR products from the titration experiment described above and supplement them with additional MgCl₂ and perform a direct comparison of mean current generated by the assays to test this theory. The mean currents generated by the samples supplemented with additional MgCl₂ (Fig 3.9) showed an increase in signal up until the point where 3.2mM MgCl₂ (2.4mM + 0.8mM supplemented) was present in the reaction with mean currents of 17.5 ± 2.2 nA, 50.3 ± 5.2 nA and 82.2 ± 10.4 nA being produced. The signal then decreased to 64.9 ± 4.1 nA when 4.0mM MgCl₂ was present in the reaction (3.2mM \pm 0.8mM MgCl₂ added post-PCR). The mean currents produced by samples which were supplemented with MgCl₂ produced similar levels of current at 1.2mM and 1.6mM MgCl₂: 17.5 ± 2.2 nA and 50.3 ± 5.2 nA respectively compared with currents of 19.7 ± 2.5 nA and 57.5 ± 4.1 nA produced by the equivalent, unsupplemented samples. However, when the samples containing 2.4mM and 3.2mM MgCl₂ in the PCR reaction were supplemented with MgCl₂ they produced currents of 82.2 ± 10.4 nA and 64.9 ± 4.1 nA respectively, compared with currents of 119.7 ± 13.6 nA and 145.3 ± 12.9 nA produced by the equivalent, unsupplemented samples. This result clearly

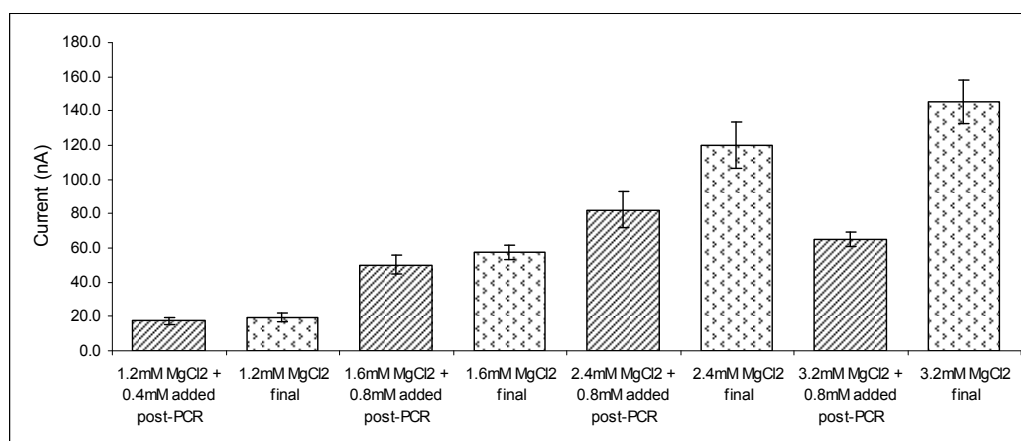


Figure 3.9. Mean currents produced by samples with additional MgCl₂ added post-PCR compared to samples with no additional MgCl₂. The MgCl₂ concentration has been calculated following dilution of 20µl PCR product with 5µl probe-T7 exonuclease. The DNA-free samples all produced zero current and are not shown. Error bars represent SD (n=3).

indicated that addition of MgCl₂ post-PCR in fact had a detrimental effect on the level of current produced. This was an unexpected result and one that could not be readily explained. Further investigation into these phenomena was not carried out due to time constraints.

The general trend was that increasing MgCl₂ concentration increased the current produced in the assay. It was unclear whether this effect was due to improved amplification in the PCR or improved digestion of bound probe – or a combination of these processes. Addition of MgCl₂ after PCR was complete actually produced lower currents compared to untreated samples with the same initial amount of MgCl₂. This happened in spite of the fact that the total MgCl₂ concentration had not exceeded the optimum amount for T7 activity and was in fact closer to this level than the untreated samples. It is likely that further investigation would cast light on which process is the more reliant on MgCl₂ and establish a way to optimise the amount of Mg²⁺ ions available to each stage of the reactions. For the purposes of developing and testing the probes a concentration of 3mM MgCl₂ added to PCR was used.

3.3 Ferrocene-labelled probes

3.3.1 Probe Optimisation.

In the assay, the ferrocene labelled probe is added to the PCR products alongside T7 exonuclease and the hybridisation reaction and the digestion of dsDNA occur simultaneously at 37°C which is the optimum temperature for T7 exonuclease activity. The signal generated by the assay is dependent on a number of factors, the most important of which is probe concentration - if too little probe was added this would limit the amount of ferrocene liberated by exonuclease digestion and lead to generation of a suboptimal signal, if too much was added then the background signal produced by oxidation of undigested probe would mask the signal produced by samples with target DNA. Performing experiments to optimise probe concentration for the fungal probe assay provided an opportunity to study how probe concentration affected the current produced by the assay.

Initial experiments at Atlas had been carried out using 3µM of probe. In order to find the optimum probe concentration for the fungal detection assay a titration experiment was set up using a range of probe concentrations from 500nM to 5µM. The mean currents produced are shown in figure 3.10. As expected there was an increase in current as the probe concentration increased and a plateau in current was observed at probe concentrations of 3.5µM and above. This was also true for the DNA-free samples which measured the background current produced due to oxidation of undigested probe molecules in the reaction. Although their distribution was a little more variable the signal reached a plateau at probe concentrations of 3.5µM and above. At all concentrations tested the samples with target present produced higher levels of current than the same amount of undigested probe with the ratio of signal to background varying between 2.4 to 4.4; the probe concentration that gave the highest ratio was 2.5µM and this concentration was incorporated into the initial assay conditions.

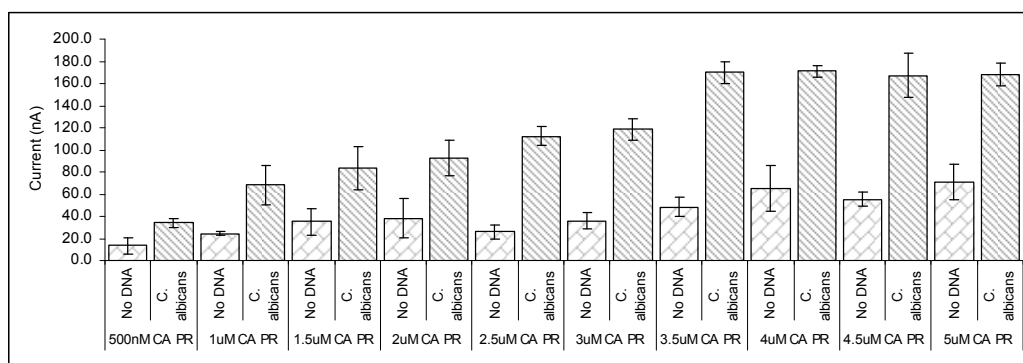


Figure 3.10. Mean currents produced in the probe titration assay using probe CA PR. Error bars represent SD (n=4).

3.3.2 Assay signal vs. background signal

The results in fig 3.10 raised an interesting question – why is the value of current less when an undigested probe is present in the reaction compared to the same amount of digested probe? In other words, what is the mechanism by which the assay distinguishes between samples with no target DNA present and assays with target? The working model of the assay assumes that there are two species of molecule capable of producing current. The first are the undigested ferrocene-oligonucleotide molecules and the second are the ferrocene-mononucleotide molecules produced following digestion of bound probe molecules by T7 exonuclease. The model predicts that there are a number of potential reasons which could account for the observed difference in current. The first is that oxidation of ferrocene is dependent on the surrounding atoms and the ferrocene label at the end of an oligonucleotide would exhibit less efficient transfer of electrons due to interference from the molecules of DNA. Unfortunately such considerations are the remit of electrochemists to study and experimental investigation of this possibility was considered beyond the scope of this work.

The second theory relies on the importance of diffusion of electro-active molecules to the electrode surface in the production of current. It postulates that the smaller ferrocene-mononucleotides can diffuse more efficiently through the reaction medium than the bulkier, intact ferrocene-oligonucleotides. Since the current produced is dependent on the number of ferrocene molecules present at the electrode surface and there will be more of

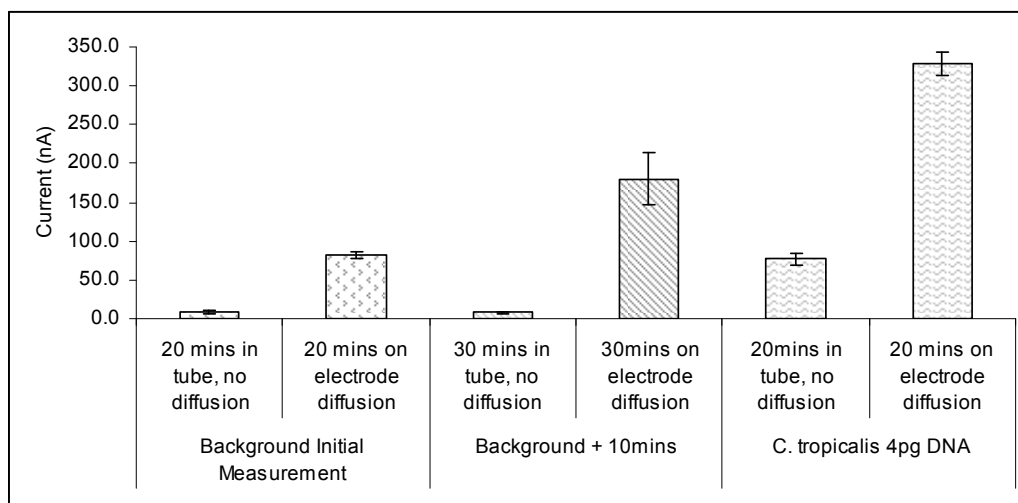


Figure 3.11. Mean currents produced by assays testing the effect of on-electrode incubation with probe CT PR. The error bars represent SD (n=3).

the fast diffusing ferrocene-mononucleotide molecules present compared to undigested probe molecules over the same time period, the samples with digested products produce a higher signal. If diffusion is indeed a key factor for current production then allowing the undigested probe extra time to diffuse to the electrode surface should result in an increase in current compared to time zero. An assay was set up in which the probe hybridisation and T7 digestion of some of the PCR products was carried out directly on the electrode for 20mins before measurements were taken and some products were incubated at room temperature (which allowed extra time for diffusion to occur) for a further 10mins before currents were measured. The currents produced by these samples were compared to samples which had the hybridisation-digestion reaction occur in a test tube and the products applied to an electrode and measured immediately. The results are shown in fig 3.11 and showed that there was a connection between increased incubation on the electrode and increased current. Target-free background samples that were incubated in tubes and electrochemically analysed as soon as they were pipetted onto the electrode produced a mean current of $8.7 \pm 2.2\text{nA}$ compared a value of $82.2 \pm 4.7\text{nA}$ for samples that had been incubated onto an electrode for 20mins. The mean currents obtained following incubation of samples for a further 10mins at room temperature showed a significant increase in mean current for the samples incubated on electrodes with a

mean current of $180.3 \pm 33.9\text{nA}$ being produced ($P < 0.05$). The samples incubated in tubes produced a mean of $8.0 \pm 1.1\text{nA}$. When samples containing target DNA were incubated in tubes they produced a mean current of $76.9 \pm 8.2\text{nA}$ compared to $328.0 \pm 15.6\text{nA}$ for samples incubated for 20mins on the electrode ($P < 10^{-5}$).

The results demonstrated that incubation of samples on the electrode had a highly significant effect on the value of current produced. This effect was likely to be due to increased diffusion of ferrocene-labelled molecules to the electrode surface. The times allowed for the products of the reaction to diffuse to the electrode surface were still not enough to allow diffusion of all of the undigested ferrocene-labelled oligonucleotides and there was still a difference between the background signal and the signal produced when target DNA was present. Fig 3.11 shows that it took 20mins of on electrode incubation (i.e. 20mins of diffusion) with undigested probe to produce the same level of current as digested products applied to the electrode and measured immediately.

If the difference in current between the intact probe vs. digested probe was entirely due to diffusion then it follows that, given enough time for undigested products to diffuse to the electrode surface, the background current would be equal to the current generated by digested products. This experiment was not attempted but would confirm the extent to which diffusion plays a role in generation of signal in the assay.

3.4 T7 exonuclease digestion

T7 exonuclease is a double stranded DNA-specific exonuclease which digests dsDNA in a $5' \rightarrow 3'$ direction to produce mononucleotides. Prior experimentation by Atlas Genetics researchers had led to the standard practice of using 10U of T7 exonuclease in the hybridisation/digestion stage of the assay. While the exonuclease activity was reported as being specific only to dsDNA it was prudent to confirm this experimentally as any ssDNA activity could be responsible for digestion of unbound probe and the production of background signal in target-free reactions and such a finding would be of

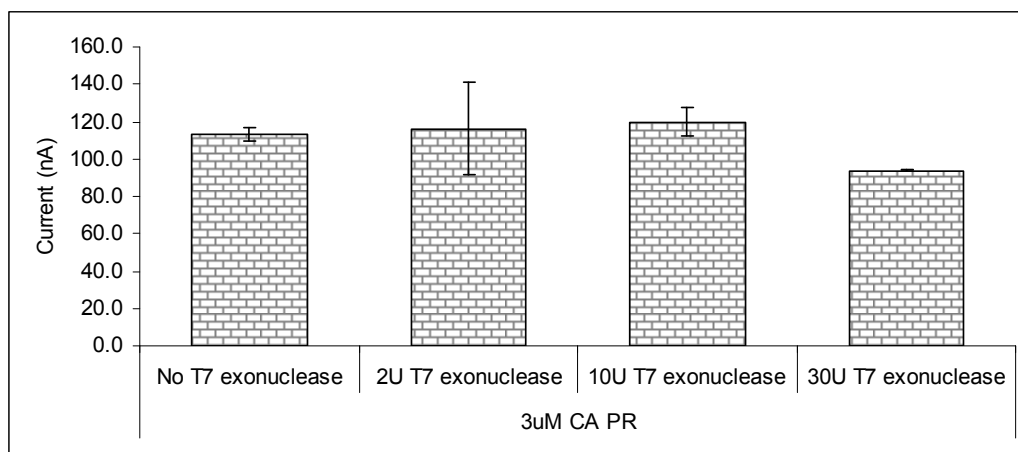


Figure 3.12. Mean currents produced in assays testing the ability of T7 exonuclease to digest unbound probe in the Illustra *Taq* PCR buffer. Error bars represent SD (n=4).

consequence to the assay. Assays were set up with 3 μ M CA PR added to the 1X IllustraTaq PCR buffer solution and different amounts of T7 exonuclease added to the reactions. The background signal was provided by assays with no added T7 exonuclease and was measured to be 112.9 ± 3.6 nA (fig 3.12). Assays which had 2U, 10U and 30U of T7 exonuclease added gave mean currents of 116.2 ± 24.9 nA, 120.0 ± 7.6 nA and 93.9 ± 0.7 nA respectively. There was no significant difference between the mean currents produced when no T7, 2U of T7 and 10U of T7 were present in the assay. When 30U of T7 was present the mean current was significantly lower than the background value ($P < 0.001$). One possible reason for this was that a change in the reaction constituents caused by the glycerol and other compounds present in the T7 exonuclease storage buffer were present in sufficient quantities to inhibit the diffusion of the electroactive molecules. This possibility is discussed in more detail in section 6.X. Regardless, the results demonstrated that there was no ssDNA activity associated with T7 exonuclease and it was not involved in production of background current.

3.5 Reduced background due to alternative reaction conditions

Researchers at Atlas Genetics had optimized a new set of PCR conditions for the assay that minimized the background signal reducing it to zero. The new conditions involved using a PCR buffer made in-house (see chapter 2 for details) and a different *Taq* DNA polymerase (Jumpstart, Sigma).

Figure 3.13 shows example currents of assays using the old PCR conditions vs. the new conditions to demonstrate the zero background current.

The reasons for the reduction of the background signal are not known. It was noted that the Jumpstart enzyme storage buffer contained a number of surfactants which may have affected the reaction matrix, inhibiting the diffusion of the undigested probe to the electrode, although the value of current obtained with target-containing samples was not diminished, as would be expected if this was the sole reason for the removal of the background signal. The effect of surfactants was briefly explored and the findings are presented in section 7.2 though the surfactants used were not of the same type or at the same concentration as would have been in the Jumpstart reaction conditions so the results are not comparable. Although it would have been of interest to thoroughly investigate this issue, understanding every aspect of the assay was not absolutely necessary (nor could enough resources be channelled into a proper investigation) and the fact remained that the output had been vastly improved by this modification.

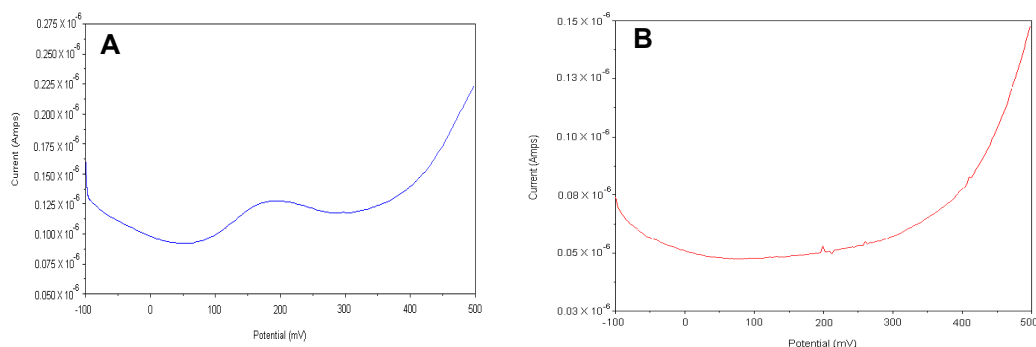


Figure 3.13. Voltammograms comparing the background signal produced by (A) the old PCR conditions (using Illustra *Taq* DNA polymerase) vs. (B) the new PCR conditions (using Jumpstart DNA polymerase).

3.6 Bioinformatics analyses.

3.6.1 Phylogenetic analysis

It was considered advantageous to the project if a rapid means of assessing ITS2 regions of non-target species for potential cross-reactivity with the species-specific probes could be developed. Constructing phylogenies based on ITS2 sequence data was a potential means to do this but was far from a foolproof solution. The problem was that phylogenies makes use of the sequence data as a whole and the inferred relationships may not reflect the presence of localized sequences of high similarity that may represent a potential 'false' probe binding site. Figure 3.14 shows one such phylogeny based on ITS2 data from 36 species which places *C. glabrata* relatively distantly from *C. famata* and *C. guilliermondii* – the two cross-reacting species. If potential targets for cross-reactivity with the CG PR probe were to be chosen based on the data in figure 3.12 then the species *C. bracarensis*, *C. kefir* and *Saccharomyces cerevisiae* would be the most likely suspects. While *C. bracarensis* and *S. cerevisiae* had not yet been tested, it had been shown that *C. kefir* did not cross react with CG PR. A more specific method was therefore required to reliably identify localized areas of sequence in non-target species which had high similarity to probe binding sites and might cause a false positive result to be obtained.

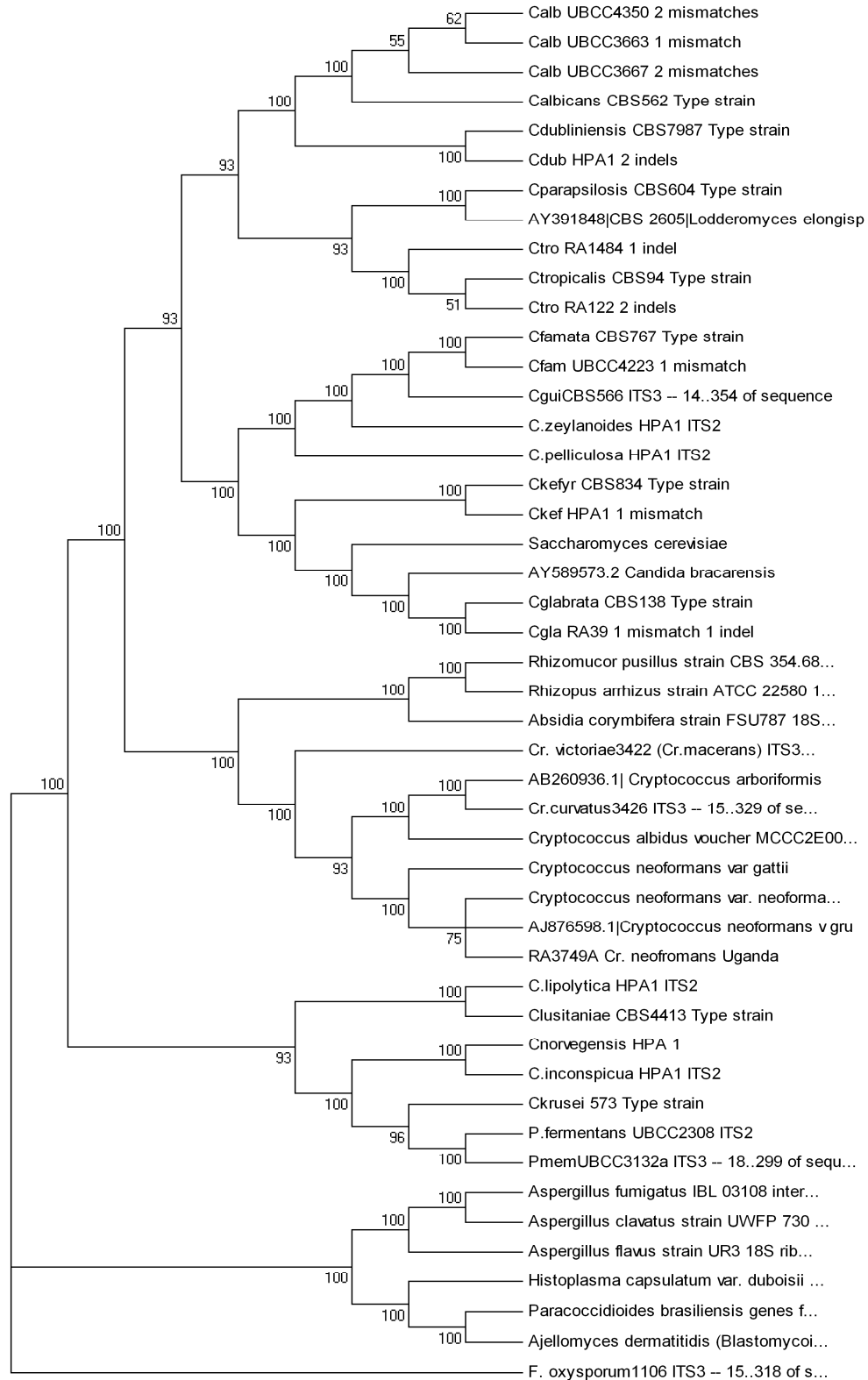


Figure 3.14. Maximum parsimony phylogeny based on a matrix of 47 fungal ITS2 sequences from 36 species. The consensus tree is shown.

3.6.2 Use of the BLAST algorithm.

A more effective method was to use the BLAST algorithm to query a database of all of the fungal ITS2 sequences from the matrix used to construct fig 3.14 with another database made of all of the probe sequences. The output from the BLAST search indicated all of the species which possessed localized regions of sequence with high similarity to the probe sequences for all of the species. The results (table 3.1) were much easier to interpret than a phylogeny and could be used to identify the potential cross-reacting binding site and check it against the entirety of the probe sequence to ascertain the likelihood of cross-reactivity occurring. The results identified a number of species were that could potentially cross-react with CG PR: *C. guilliermondii*, *C. famata*, *S. cerevisiae*, *C. pelliculosa*, *C. bracarensis* and *C. zeylanoides*. *C. guilliermondii* and *C. famata* had already been found to cross

Table 3.1. Part of the BLAST output of the CG PR probe when queried against the database of fungal ITS2 sequences.

Probe	Query Species	Total matches	E-value	Bit Score
CG_PR	Cglabrata_RA39_1mis_1indel	25	1.90E-10	50.05
CG_PR	Cglabrata_CBS138	25	1.90E-10	50.05
CG_PR	Scerevisiae	15	1.80E-04	30.23
CG_PR	Cbracarensis_AY589573.2	15	1.80E-04	30.23
CG_PR	Czeylanoides_HPA1	17	0.674	18.33
CG_PR	Cpelliculosa_HPA1	17	0.674	18.33
CG_PR	Cfamata_UBCC4223_1_mis	17	0.674	18.33
CG_PR	Cfamata_CBS767	17	0.674	18.33
CG_PR	Cguilliermondii_CBS566	17	0.674	18.33
CG_PR	F.oxysporum_1106	8	2.7	16.35
CG_PR	F.oxysporum_1106	7	10.5	14.37
CG_PR	Cr.albidus_voucher_MCCC2E00	8	2.7	16.35
CG_PR	Rhizomucor_pusillus_CBS354.68	8	2.7	16.35
CG_PR	Absi.corymbifera_FSU787	8	2.7	16.35
CG_PR	Ctropicalis_RA122_2indels	8	2.7	16.35
CG_PR	Ctropicalis_RA122_2indels	7	10.5	14.37
CG_PR	Ctropicalis_RA122_2indels	7	10.5	14.37
CG_PR	Ctropicalis_RA1484_1indel	8	2.7	16.35
CG_PR	Ctropicalis_RA1484_1indel	7	10.5	14.37
CG_PR	Ctropicalis_RA1484_1indel	7	10.5	14.37

react with the CG PR probe (see section 4.3.3 below) but the other species had not been examined.

The e-value and bit score placed *S. cerevisiae* and *C. braccarensis* above the other species even though the actual number of matched base pairs was lower in the former species. This raised the question of which statistic was the most useful for predicting cross-reactivity and to answer this, assays with the CG PR probe were performed using PCR products amplified using DNA from *S. cerevisiae*, *C. pelliculosa* and *C. zeylanoides* (DNA from *C. braccarensis* was not available for the experiment).

The mean currents are shown in figure 3.15 and indicate that cross reactivity was observed between the species tested and CG PR. The background current was measured at $25.3 \pm 7.2\text{nA}$ while the mean currents for the non-target species were $52.3 \pm 2.3\text{nA}$ (*C. zeylanoides*), $67.0 \pm 7.8\text{nA}$ (*C. pelliculosa*) and $49.2 \pm 3.2\text{nA}$ (*S. cerevisiae*) and these compared to a signal of $82.9 \pm 6.7\text{nA}$ obtained by the assays with *C. glabrata*. These results were confirmation that a BLAST query could identify species likely to cross-react with a probe. Table 3.1 showed that the potential probe binding site in *S. cerevisiae* had the lowest e-value out of all of the species tested, while the data for the binding sites present in *C. pelliculosa* and *C. zeylanoides* were the same. However, *C. pelliculosa* produced the highest mean signal out of the three species while the values obtained by *C. zeylanoides* and *S. cerevisiae* were very similar. It was possible that differences in the production of amplicon in the PCR stage was a factor in the production of different levels of current. In assays with amplicon from these species, particularly as the amount of template DNA added to each reaction was not standardized. Hence no solid conclusions can be made as to the predictive power of the BLAST search results in respect of the level of signal in the assay, only that it can indicate potential cross-reactivity which is still of immense use in itself.

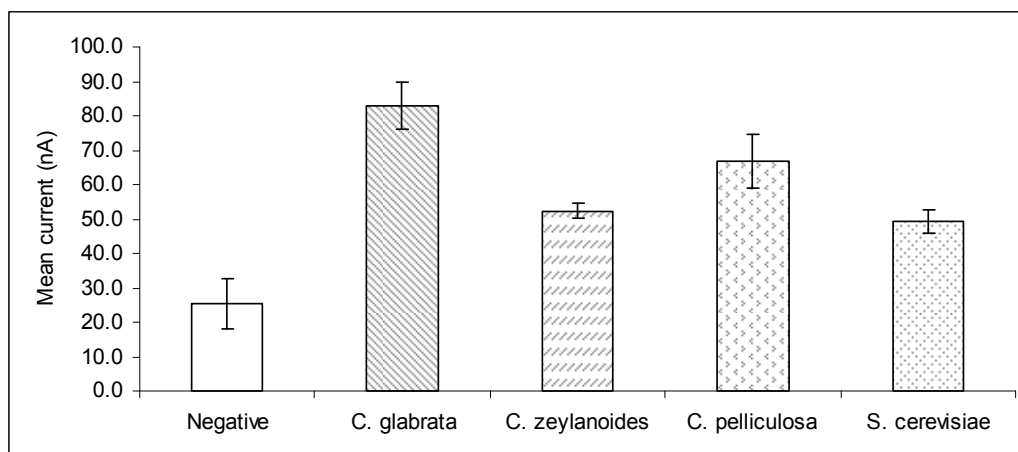


Figure 3.15. Mean currents produced by assays using the CG PR probe to detect DNA from potentially contaminating species. Error bars represent SD (n=4).

Of the other probes that were used to query the ITS2 matrix CA PR3 and CK PR generated results that suggested potential for cross-reactivity with non-target species; a region within the *C. dubliniensis* ITS2 sequence was identified which shared 14bp (70% similarity) with the CA PR3 probe and regions within the *C. inconspicua* and *P. fermentans* ITS2 sequences were identified which shared 10bp (40% similarity) and 12bp (48% similarity) with the CK PR probe. Section 4.3.2 shows the results obtained when the CA PR3 probe was tested with a number of *Candida* species including *C. dubliniensis* and demonstrates that no cross-reactivity was observed. Further assays were performed with the CK PR probe and the potentially cross-reacting species; as well as the usual DNA-free negative and target-containing PCR positive, a non-target negative sample was included in the form of assays performed on products amplified from *C. tropicalis* DNA. The results produced were much lower than expected for all species tested (fig 3.16) with the mean current produced by the target-containing samples reaching only $10.1 \pm 0.8\text{nA}$. The background signal produced by the DNA-free sample was measured at $5.1 \pm 1.0\text{nA}$ while the non-target negative produced a signal of $3.8 \pm 1.2\text{nA}$. The potentially cross-reacting species, *P. fermentans* and *C. inconspicua* produced mean currents of $4.6 \pm 0.6\text{nA}$ and $6.0 \pm 2.6\text{nA}$ respectively. These values were not significantly different to the background, suggesting that cross-reactivity with these species was not likely to be a problem. The results should be treated with caution, however, due to the anomalous values of

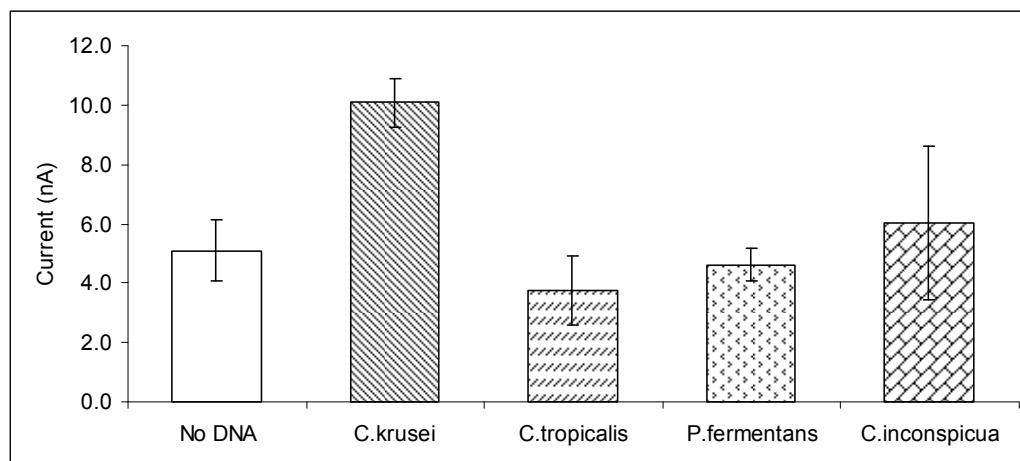


Figure 3.16. Mean currents produced by assays with probe CK PR and a number of non-target species. Note the anomalously low values of current produced by all samples in the assays. Error bars represent SD (n=4).

current that were obtained. Replication and possible further experimentation were not able to be performed due to time constraints.

The results of the BLAST query performed using the other probes (including the redesigned *C. glabrata* specific probe, CG PR3) indicated that cross-reactivity was unlikely to occur with any of the species represented in the ITS2 sequence matrix (data not shown).

3.6.3 Use of BLAST query output to inform probe design.

The experiments described above for the probe CG PR provide some support for the notion that cross-reactivity is a potential problem only when the percentage of matching bases is above a certain percentage, but that is not the whole story. As stated above, the BLAST query identified that the CA PR3 probe had a 70% similarity to part of the *C. dubliniensis* ITS2 region, which was more than the similarity of CG PR to *C. guilliermondii* or *C. famata*, but the CA PR3 probe did not cross-react. This showed that it was not just the number of bases that was important, but the position of matching bases played a major role in cross-reactivity. For example, in the *C. dubliniensis* ITS2 there is a region in which 14 out of 20 bases match the CA PR3 probe binding site but, crucially, the first three bases at the 5' end were mismatched. It was thought likely that this is what prevented the CA PR3 probe giving a

positive signal with *C. dubliniensis*. Conversely, in the cross-reacting species *C. guilliermondii* and *C. famata* there are 17 and 18 matched bases to the CG PR probe binding site and the first seven of the 5' bases are an exact match to the sequence of CG PR and these non-target species produce a relatively high current when assayed with this probe (see chapter 4). Figure 3.17 shows the potential binding site in the ITS2 regions of *C. pelliculosa* and *C. zeylanoides* where it can again be seen that the first six or seven of the 5' bases respectively are an exact match to the probe sequence.

The observations made above suggest that it is the identity of the initial bases at the 5' end of the probe sequence that determine whether cross-reactivity is likely to occur or not. The 'total matches' statistic from the BLAST output is probably the most useful statistic to use to decide if a probe may cross-react with a non-target species, since a greater number of matching nucleotides increases the probability that there will be matches at the 5' end of the probe and non-target binding site. Although the conclusion is by no means cast-iron there is compelling evidence in support of it and, if it can be more strongly supported, it would allow greater freedom in designing probes for new target species as only the first few bases need be mismatched to non-target species in order to ensure specificity of the probe in the assay.

The use of the BLAST algorithm to query the fungal ITS2 sequence matrix with the probe sequences was an extremely useful method to establish non-target species that could potentially give false positive results with a given probe and the sequence data could then be examined further to examine the exact nature of the similarities.

<i>C. pelliculosa</i>	5' TAGGTTCTTCCAACCTCGTTATATCA 3'
CG PR	5' TAGGTTTACCAACTCGGTGTTGAT 3'
 <i>C. zeylanoides</i>	 5' TAGGTTTATCCAACCTCGTTGACCAG 3'
CG PR	5' TAGGTTTACCAACTCGGTGTTGAT 3'

Figure 3.17. Potential probe binding sites of *C. pelliculosa* and *C. zeylanoides* to the *C. glabrata*-specific probe CG PR.

3.7 Design of the panfungal probe, PanF PR using bioinformatics.

As well as screening species specific probes to detect potential cross-reactivity, the BLAST strategy could be used to rapidly screen probes designed to detect multiple species. When attempting to design a probe capable of detecting DNA from any fungal species this approach was used to confirm the ability of potential panfungal probe sequences, which had been designed by eye, to include all of the pathogenic fungal species for which sequence data had been acquired and at the same time confirm that it should exclude non-target species i.e. human.

The probe, designated PanF PR, was designed to the 18s rRNA gene and was initially designed by eye using a matrix of sequences from mainly pathogenic yeasts but that also included sequences from pathogenic *Aspergillus* species and the human 18s rRNA sequence. It was not easy to locate a suitable conserved sequence in the rRNA genes and the probe sequence was not a perfect match to any of the sequences, but was designed with as few mismatches as possible. In order to confirm its similarity to a wider range of fungi more 18s sequences from pathogenic fungi were incorporated into the sequence matrix as well as sequences from eukaryotic pathogens of humans that may be found in blood: *Plasmodium vivax*, *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* and this expanded sequence database (26 fungal + 4 non-fungal sequences) was queried using the potential PanF PR sequence. Part of the BLAST output is shown in table 3.2, showing all 26 fungal sequences giving matches which ranged from 22 matching bases (100% similarity) to 17 matches (77% similarity). The only non-target species that was included in the output was for *Plasmodium vivax* which produced a weak hit of 11bp on the opposite strand (data not shown). The probe binding sites for the species included in the query matrix are shown in fig 3.18. One of the most common mismatches was at the second position which was a guanine residue in the probe sequence but was an adenine residue in 14 out of the 26 fungal species. Since this polymorphism was present at the critical 5' end of the target site there was a chance that the probe wouldn't bind efficiently. However, as mentioned previously, the CK PR probe had a greater number of mismatches at its 5' end but was still capable

Table 3.2. Output from the BLAST search performed using the PanF PR probe as a query sequence, sorted by decreasing number of base pair matches.

Species	Total matches	% total matches	Species	Total matches	% total matches
<i>Lomentospora prolificans</i>	22	100	<i>Candida_zeilanoide</i> s	21	95
<i>Candida_inconspicua</i>	21	95	<i>Cryptococcus_curvatus</i>	21	95
<i>Candida_pelliculosa</i>	21	95	<i>Ajellomyces_capsulatum</i>	21	95
<i>Candida_glabrata</i>	21	95	<i>Penicillium sp</i>	20	91
<i>Candida_parapsilosis</i>	21	95	<i>Scopulariopsis brevicaulis</i>	20	91
<i>Candida_tropicalis</i>	21	95	<i>Candida_lusitaniae</i>	20	91
<i>Candida_albicans</i>	21	95	<i>Yarrowia_lipolytica</i>	20	91
<i>Kluyveromyces_marxianus</i>	21	95	<i>Cryptococcus_neoformans</i>	20	91
<i>Pichia_guilliermondii</i>	21	95	<i>Aspergillus_terreus</i>	19	86
<i>Candida_dubliniensis</i>	21	95	<i>Aspergillus_fumigatus</i>	19	86
<i>Debaryomyces_hansenii</i>	21	95	<i>Fusarium_solani</i>	19	86
<i>Issatchenkia_orientalis</i>	21	95	<i>Saksenaea_vasiformis</i>	17	77
<i>Saccharomyces_cerevisiae</i>	21	95	<i>Rhizopus_oryzae</i>	17	77

of differentiation between target and non-target containing samples. Given the difficulty of finding conserved panfungal sequences it was decided to obtain the probe and test it experimentally. In addition to the probe sequence, fungal specific primers had to also be designed to amplify the target site. Again, there was difficulty in designing these to be panfungal but appropriate sequences were found which differed sufficiently at their 3' ends to exclude the non-target species from being amplified while including all fungi for which sequence data had been acquired. A number of primers were able to be designed by eye and after pair-wise primer trials the pair 18sF2 and 18sR4 (table 2.2) were determined to be the most successful and were used in assays with the PanF PR probe.

PanF_PR	GGTGATTCAT AATAACTT-- ---TTCG
<i>C. albicans</i>	.A..... --
<i>C. glabrata</i>	.A..... --
<i>C. parapsilosis</i>	.A..... --
<i>C. tropicalis</i>	.A..... --
<i>C. krusei</i>	.A..... --
<i>C. dubliniensis</i>	.A..... --
<i>C. lusitaniae</i>	.A..... --G..
<i>C. famata</i>	.A..... --
<i>C. kefyr</i>	.A..... --
<i>C. guilliermondii</i>	.A..... --
<i>P. anomala</i>	.A..... --
<i>C. zeylanoides</i>	.A..... --
<i>C. lipolytica</i>T... --G..
<i>C. inconspicua</i>	.A..... --
<i>Saccharomyces cerevisiae</i>	.A..... --
<i>Aspergillus fumigatus</i>A.... --AA..
<i>A. terreus</i>C.... --AA..
<i>Cryptococcus neoformans</i>A.... --C..
<i>Cr. curvatus</i> --C..
<i>Fusarium solani</i>G.....C-- --C..
<i>Saksenaea vasiformis</i>C.... ..T... --AG.T
<i>Rhizopus oryzae</i>A.... ..T.A-- --AG..
<i>Penicillium sp.</i> --CA..
<i>Ajellomyces capsulatum</i> --G..
<i>Scopulariosis brevicaulis</i>G-- --G..
<i>Lomentospora prolificans</i> --
HumanC..TA G.....C.CG --GG.C
<i>Plasmodium vivax</i>	A.G..A.T.. ..C..AGAAG TGACA..
<i>Trypanosoma brucei rhodesiense</i>	.T...C...A TTC.TTCCG- ---G..
<i>Trypanosoma brucei gambiense</i>	.T...C...A TTC.TTCCG- ---G..

Figure 3.18. Probe binding sites in the 18s rRNA gene of 26 fungal species + 4 non-fungal species.

3.8 Conclusions.

The basic model of the assay was introduced and experimental results presented which validated this model, at least partially. The importance of the PCR amplification step or, more specifically, the PCR primers, for reliable and satisfactory production of signal in the assay was demonstrated and this was of major importance for the work into assay sensitivity (chapter 5). A likely mechanism for the differentiation between signal and background was also presented which accounts for the results produced by the assay, though it is not likely to be only mechanism which exercises an effect on the assay signal as there are a number of factors at work which may affect the assay. Nonetheless, the assay has been shown to work in principle and the reaction

conditions were able to be optimised to yield currents with a relatively good signal:noise ratio.

The occurrence of false positive results with the probes was also introduced. Thorough initial screening of probes is a necessity to avoid the possibility of cross-reactivity leading to false positives in later experimentation. Phylogenetic reconstructions can indicate sister species based on DNA sequence information but this does not necessarily correlate to likelihood of cross-reactivity. A more robust bioinformatic approach to probe design was therefore developed that could be employed to rapidly screen probes to ensure specificity. Furthermore, it was shown that this method could also inform novel probe design by ensuring that target species could be detected. A panfungal probe was able to be designed using this method. Having established the model and experimental conditions for the assay the next stage in the development was to test the species-specific probes and the newly-designed panfungal probe experimentally to ensure their specificity and sensitivity.

Chapter 4 - Specificity of the probes.

4.1 Introduction.

For a diagnostic test it is essential that probes be specific to their target – detection by a probe of non-target species is unacceptable as it represents a failure of the probe to detect a single target species and, particularly in the case of diagnosis of invasive *Candida* infections, could lead to inappropriate antifungal therapy being administered with possibly life-threatening consequences. For an end-point PCR hybridization assay such as the Atlas electrochemical assay the specificity lies in the choice of DNA target and can be built into either or both the primers used in PCR amplification and the probe molecule.

The remit of the species-specific part of the detection assay was to be able to detect DNA from the five most clinically relevant species of *Candida*. There are many potential DNA regions that could be targeted for species-specific probes but it was decided that the ITS2 region of the rDNA genes, with its repeated nature and high degree of interspecies variability, would provide the necessary specificity for the assay. Probe sequences specific for *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* (CA PR, CP PR, CG PR, CT PR and CK PR respectively) that targeted this region were acquired from Shin *et al.* (1999). PCR primers designed to the conserved rRNA genes have been used for phylogenetic studies by other researchers (e.g. White *et al.*, 1990) and the fungal universal primers ITS3 and ITS4, which amplify the ITS2 region (fig 4.1), were used to amplify target DNA for detection with the assay. Species-specific primers could potentially have been designed and would have added another layer of specificity to the assay, but would have made it more difficult to multiplex the assay. The use of generic primers would also allow additional probes for additional target species to be easily incorporated into the existing assay without having to optimize PCR conditions for each primer-probe combination.

The first challenge to the assay, therefore, was to test the universal nature of the primers alongside the specificity of the probes. It was of paramount

C._albicans_GQ280318.1	<u>CGCATCGATG</u>	<u>AAGAACGCAG</u>	<u>CGAAATGCGA</u>	TACGTAATAT	GAATTGCAGA	TATTCGTGAA	TCATCGAATC	TTGAACGCA
C._parapsilosis_EU871507.1A.....
C._glabrata_AB305094.1G.	AT.C.....
C._tropicalis_GQ376071.1
C._krusei_L47113.1C..G.G.C	C..-.....G.T	C.....
C._albicans_GQ280318.1	CATTGCGCCC	TCTGGTATTC	CGGAGGGCAT	GCCTGTTTGA	GCGTCGTTTC	TCCCTCAAAC	-CGCTG--GG	TTTGGTGTG
C._parapsilosis_EU871507.1T.....	..AA.....A.....	-.TTC.--.
C._glabrata_AB305094.1G.....A....	CTT.....	A..T..--T.AG..
C._tropicalis_GQ376071.1T.....	..AA.....A....	-.C.C.--.
C._krusei_L47113.1	CTC..C....	...G.....	CAT..TGCG.	-GTGC.CA.A	G....G.--.
C._albicans_GQ280318.1	AGCAATACGA	CTTGGGTTT-	-----GCTT	GAAA-----G	ACGGTAGTGG	--TA-----	--AGGCGGGA	TCGC-TTTGA
C._parapsilosis_EU871507.1	...G.....-	-C.....-	-----	-----	--.....AG	-TA--.AAAC
C._glabrata_AB305094.1	..TG....T-	-C.C.T...T	GAGTTAA...TTGTA.	G.CA..TCA.	TA.GTGGGAC	ACGA...CA.	G.TTC.C.AT
C._tropicalis_GQ376071.1-	-C.A.....-	-----T..T..--	--C.T..A.	A.T--.A.TT
C._krusei_L47113.1	...GGAG..G	ACGAC..G.A	AAG--A..G.	CGG.-----	CT.CG.C.C.	CC.G-----	--AAG..AG	CGAAGC.G.C
C._albicans_GQ280318.1	CAATG-----	-----	-----	GC-TTAGG--	TCTAAC---C	AAAAACA-TT	GCTTGCGG--	-----CG
C._parapsilosis_EU871507.1	T....-----	-----	-----	.A--....--	.T.-TT---T	TTTTC..-C.	CA..-----	-----.
C._glabrata_AB305094.1	T...CTGCTG	CTCGTTTGCG	CGAGCGGCGG	.GG...ATAC	.G..TTAGGT	TTT.C..AC.	CGG..TT.AT	CTAGGGAGG.
C._tropicalis_GQ376071.1	T..GC-----	-----	-----	.AC.....-	.T..T.---G-C.	TA..TT---	-----.
C._krusei_L47113.1	.G.GC-----	-----	-----	.AAC...ACT	.T.TTT---	.GGG..G-C.	TGGC.GC---	-----..
C._albicans_GQ280318.1	GTA-ACGTCT	AC-----	-----CAC	GTATATCTTC	A-AACTTTGA	CCTCAAATCA	GGTAGGACTA	CCCCTGAAC
C._parapsilosis_EU871507.1	...--.AAAC	T.-----	-----A	AC..TCT.C.	--..A..C..
C._glabrata_AB305094.1	A..AGT.AG.	GTTTTGTGCG	TGCTGGG..G	AC.G.CG.CT	TT..G.....GT..
C._tropicalis_GQ376071.1	C..-GT.G.C	AAT.TAT...	.T.....
C._krusei_L47113.1	AG.-G..AG.	GT-----	-----TG.	..-G.CAACA	.A..GC.C..A..
C._albicans_GQ280318.1	<u>TTAAGCATAT</u>	<u>CAATAAGCGG</u>	<u>AGGAA</u>					
C._parapsilosis_EU871507.1					
C._glabrata_AB305094.1					
C._tropicalis_GQ376071.1					
C._krusei_L47113.1					

Figure 4.1. Sequence alignment of the 3' end of the 5.8s rRNA gene, the ITS2 region and the 5' end of the 26s rRNA gene of the five *Candida* target species. The binding positions of the ITS3 and ITS4 primers have been underlined.



Figure 4.2. Ethidium bromide gel picture showing PCR amplicons from 10 *Candida* species using the primers ITS3 and ITS4. DNA from each species was amplified in quadruplicate. Top row lane 1: 100bp ladder; lanes 2-5 No-DNA; lanes 6-9 *C. krusei* (~350bp); lanes 10-13 *C. glabrata* (~420bp); lanes 14-16 *C. albicans* (~340bp). Middle row lane 1: 100bp ladder; lane 2 *C. albicans* (~340bp); lanes 3-6 *C. parapsilosis* (~310bp); lanes 7-10 *C. tropicalis* (~325bp); lanes 11-14 *C. lusitanae* (~255bp); lanes 15-16 *C. famata* (~380bp). Bottom row lane 1: 100bp ladder; lanes 2-3 *C. famata* (~380bp); lanes 4-7 *C. kefyr* (~430bp); lanes 8-11 *C. dubliniensis* (~343bp); lanes 12-15 *C. guilliermondii* (~380bp).

importance that the probes not cross react with non-target species as they were the only component of the assay that provided specificity. As the precision, accuracy and reproducibility of the results produced by the assay had not yet been properly validated, there were no discrete criteria by which the success or failure of a probe was judged. Therefore a retrospective criterion was established whereby if a positive sample produced a mean current that was at least three-fold higher than the highest background it was considered to have successfully detected the target species and demonstrated specificity.

4.2 Testing the ITS3 and ITS4 primers in PCR.

The initial PCR reactions to test the ITS3 and ITS4 primers were set up using 1.5mM MgCl₂ and the Illustra Taq polymerase and buffer (materials and methods section). These conditions allowed successful amplification of the ITS2 region in 10 *Candida* species tested and an amplicon was produced

when both symmetric and asymmetric conditions were used; the size of each amplicon corresponded to the expected size based on sequence information (fig. 4.2). The high intensity of the amplicons from all 10 species visualized on the agarose gel indicated that the PCR conditions were capable of efficient amplification of the ITS2 region from fungal DNA.

4.3 Testing specificity of probes with DNA from type *Candida* strains.

The results from assays with the five species specific probes are described in the following sections. The probes performed with varying degrees of success and these experiments highlighted different problems with probe design. Of the initial probes that were trialed three produced unsatisfactory results for different reasons and the problems which were encountered are discussed in the following sections. Those that gave satisfactory results and provided reliable detection of only their target species are presented first, followed by the probes that were problematic and required further design and/or optimization.

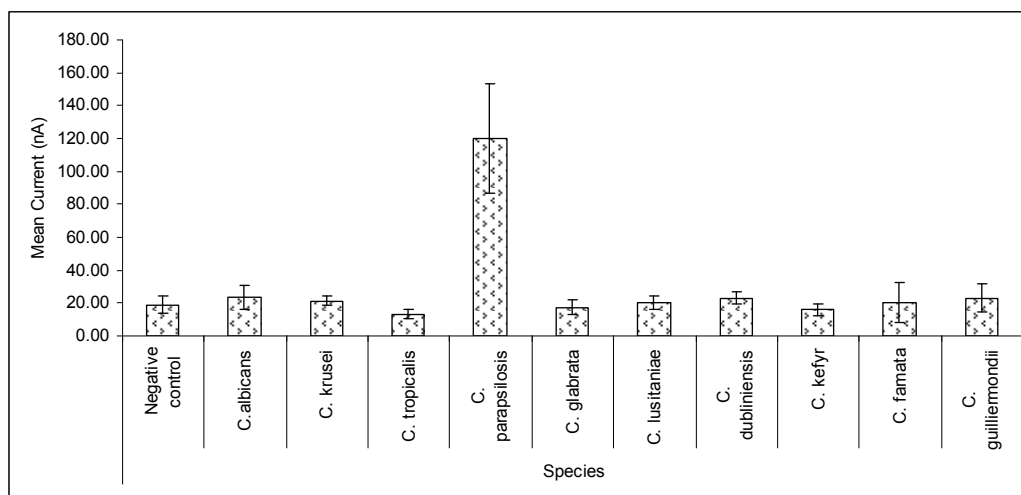


Figure 4.3. Bar graph showing the mean currents produced in assays conducted with CP PR vs. the test panel of 10 *Candida* species. Error bars represent SD (n=4).

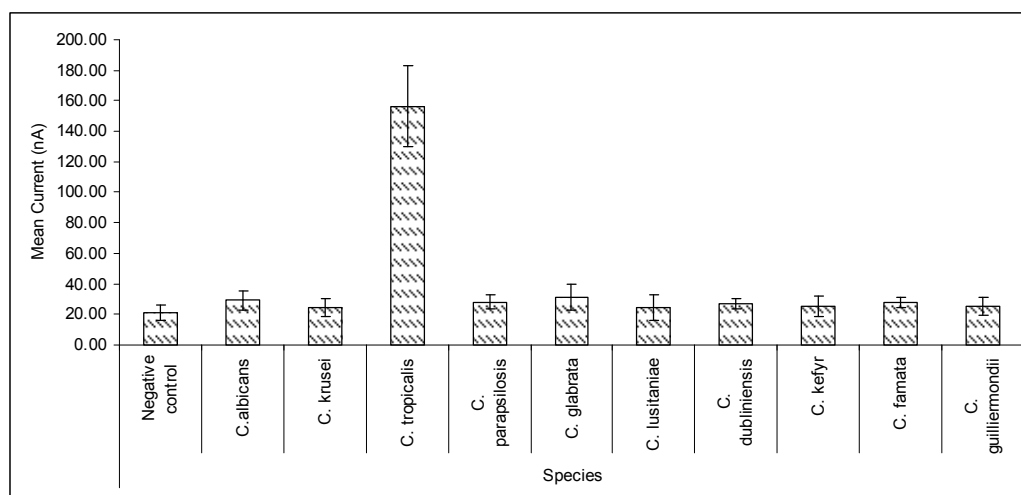


Figure 4.4. Bar graph showing the mean currents produced in assays conducted with CT PR vs. the test panel of 10 *Candida* species. Error bars represent SD (n=8).

4.3.1 Specificity of probes CP PR and CT PR.

To test the specificity of the species-specific probes experimentally the assay was performed with each of the probes using DNA extracted from 10 type strains of *Candida* species (Table 2.1) as well as a DNA-free negative sample which would provide a measure of the background current. Figure 4.3 shows the mean currents obtained with the CP PR probe using the IllustraTaq assay conditions. The mean current produced with the target *C. parapsilosis* DNA was 120.0 ± 33.5 nA compared with a background current of 19.0 ± 5.6 nA and non-target currents of between 13.3 ± 2.7 nA and 23.5 ± 7.3 nA. This satisfied the criterion of the target mean current being two to three-fold higher than the background and indicated that there was no problem as regards cross-reactivity with non-target species.

The mean results obtained with the *C. tropicalis* specific probe CT PR against the test panel of *Candida* species were similarly successful (fig 4.4). The mean current produced with target DNA was 156.5 ± 26.7 nA which compared to a background current of 21.5 ± 5.1 nA and non-target currents of between 24.2 ± 8.4 nA and 31.1 ± 8.7 nA. This allowed excellent discrimination between the background and non-target signals and the signal produced due to the presence of target DNA.

The two probes tested above worked well without further optimization and were shown to give an excellent current in the electrochemical assay with target species with no evidence of cross reactivity with non-target species from the *Candida* test panel.

4.3.2 Testing problematic probes: CA PR.

The results of the assay with the *C. albicans* specific probe, CA PR, are shown in figure 4.5. The mean current produced by the target, *C. albicans* DNA-containing sample was 58.6 ± 2.71 nA and this compared with values of between 27.5 ± 6.3 nA (*C. krusei*) and 40.5 ± 14.3 nA (*C. famata*). The background signal in the assay was 27.47 ± 4.2 nA. The values obtained with this probe did not allow significant discrimination between target and non-target containing samples the problem being that the positive signal was lower than observed with the other probes that were considered to be successful. The reason for this was unclear as the same ferrocene label had been used in the synthesis of this probe as for the others. However, the CA PR probe did differ from the others in that it had been synthesized at a different time. It was decided to have a new batch of the CA PR probe synthesized to see if there was a difference between two probes with the same DNA sequence synthesized at different times.

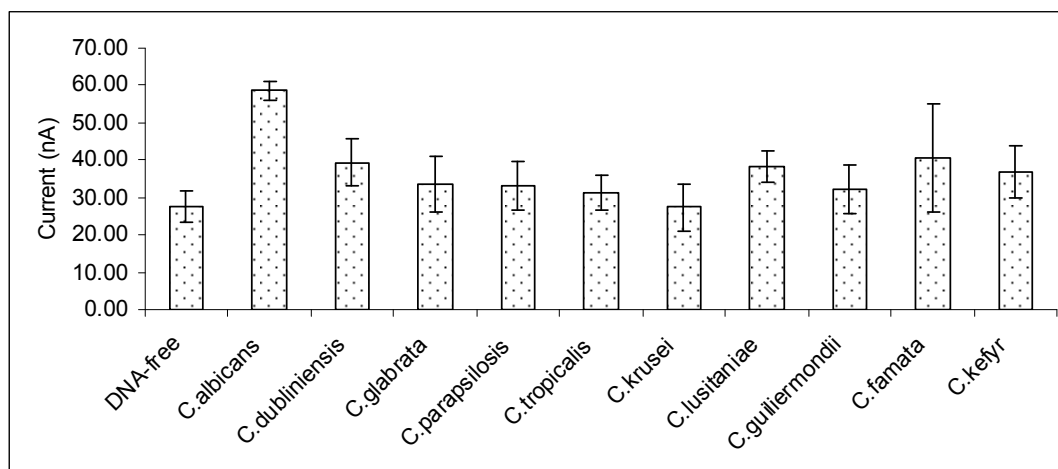


Figure 4.5. Bar graph showing the results of the electrochemical assay with the *C. albicans* specific probe, CA PR, nine other *Candida* species and DNA-free reactions. The error bars represent SD (n=4).

The newly synthesised probe (which will be referred to as CA PR NEW) was used in an assay alongside the old CA PR probe (CA PR OLD) with *C. albicans* DNA to check its performance (figure 4.6). CA PR OLD produced a mean background current of $35.5 \pm 7.5\text{nA}$ and a positive signal of $66.9 \pm 0.9\text{nA}$ compared with a background of $61.7 \pm 13.1\text{nA}$ and a positive signal of $73.0 \pm 8.0\text{nA}$ with CA PR NEW. The newly synthesized probe produced a slightly higher positive signal but at the cost of producing a background signal that was 1.7 times that of the new probe. Since both probes shared the same target site this anomaly was probably associated with the electrochemical label, though in what capacity is unknown. If it is assumed that all of the probe binds target DNA and is digested by T7 exonuclease in the assay, the results suggested that the label had not been coupled properly to the probe: when target DNA is present the probes are completely digested such that all of the label becomes free and so similar currents would be produced in the assays with target DNA, which was the case. However, when no target DNA is present, the potentially less strongly coupled label on the CA PR NEW probe may decouple from the probe and diffuse to the electrode surface, thus producing an increased background current compared with the CA PR OLD probe.

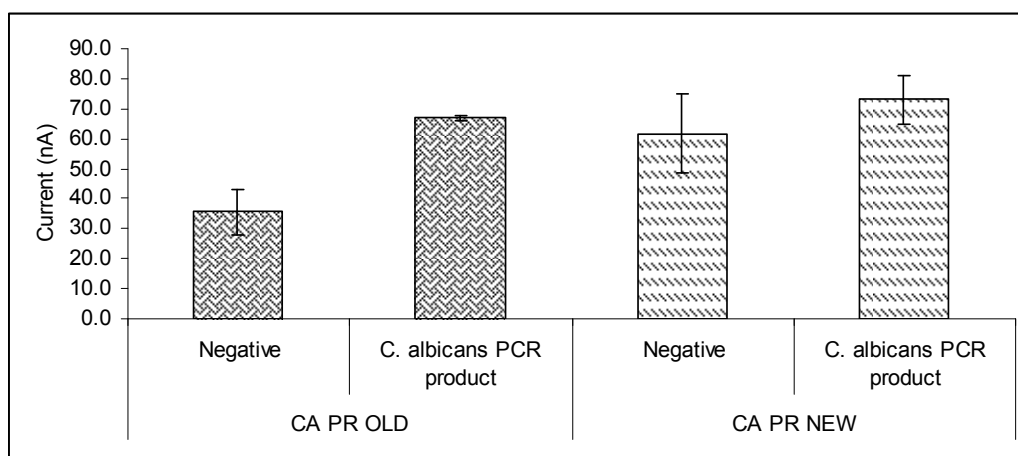


Figure 4.6. Mean results of the assay comparing the performance of CA PR OLD and CA PR NEW. Error bars represent SD (n=4).

The anomaly was investigated further using a synthetic target assay, the results of which are shown in figure 4.7. This assay did not support the hypothesis of a weakly coupled label on the CA PR NEW probe. There was a significant difference ($P<0.01$) between the mean current of $511.3 \pm 29.3\text{nA}$ produced by CA PR OLD and the mean current of $377.9 \pm 10.8\text{nA}$ produced by CA PR NEW. The difference between the DNA-free negative currents was also significant ($P<0.01$) but the values only differed by $\sim 23\text{nA}$: $105.3 \pm 4.4\text{nA}$ for CA PR OLD vs. $128.0 \pm 7.4\text{nA}$ for CA PR NEW. The relatively similar currents produced by the no-DNA negatives combined with the significantly lower signal produced by the CA PR NEW probe in an assay with synthetic target DNA did not point to the conclusion that unstable label was the cause of the strange results obtained with this probe.

The reduced signal produced by the CA PR NEW probe was unexpected and upon further investigation it was discovered that the ferrocene label used for the synthesis of CA PR NEW was not the same as the label used when CA PR OLD was synthesized. Data obtained by other researchers at Atlas Genetics suggested that the label may perform less well than the original label used to synthesise CA PR OLD and so the anomalies reported above may have been due to batch differences.

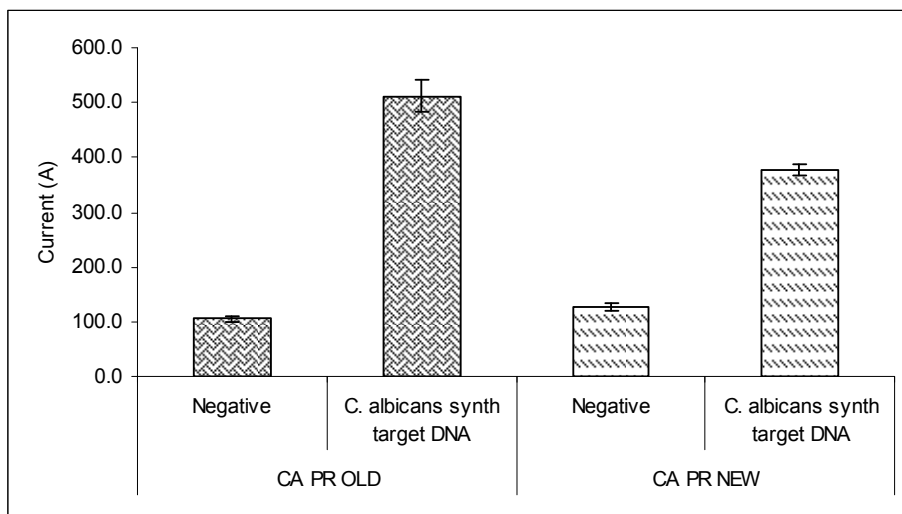


Figure 4.7. Mean currents from a synthetic target assay comparing CA PR OLD vs. CA PR NEW. Error bars represent SD (n=4).

```

C.albicans_CBS562      TCATCGAATC TTTGAACGCA CATTGCGCCC TCTGGTATTC CGGAGGGCAT GCCTGTTTGA GCGTCGTTTC TCCCTCAAAC
C.dubliniensis_CBS7987 .....

C.albicans_CBS562      CGCT-GGGTT TGGTGTGAG CAATACGACT TGGGTTTGCT TGAAAGACGG TAGTGGT--A AGGCGGGATC GCTTTGACAA
C.dubliniensis_CBS7987 .C..A..... .....T.A .....AT. ....AGAT ....-.....

C.albicans_CBS562      TGGCTTAGGT CTAACCAAAA ACATTGCTT- -GCGG-CGGT AACGTCCACC ACGTATATCT TCAAACTTTG ACCTCAAATC
C.dubliniensis_CBS7987 ..... G.....AA G....T.TC. GG....GC.. .TT.TAT... .....

C.albicans_CBS562      AGGTAGGACT ACCCGCTGAA CTTAAGCATA T
C.dubliniensis_CBS7987 .....

```

Figure 4.8. Sequence alignment showing the highly similar ITS2 sequences of *C. albicans* and *C. dubliniensis*. The position of the newly designed CA PR2 probe is shown by this style of underlining; the newly designed CA PR3 probe binding site is indicated by this style of underlining.

A further possibility was that the CA PR probe sequence itself could be the reason for the low current produced in the assay. The art of probe design for the electrochemical assay has not yet been completely perfected and it was not known exactly which features make a good probe for the system. It was decided to design a new *C. albicans* specific probe, CA PR2, synthesise it with a reliable label and test it against the original CA PR OLD probe. The design of new *C. albicans* specific probes was limited to a very small region due to a high degree of similarity of the ITS2 between *C. albicans* and *C. dubliniensis* (fig 4.8). However, enough differences were available for the design of the probe CA PR2. Figure 4.9 shows the mean currents generated in assays using synthetic target DNA between CA PR OLD and CA PR2.

The mean current produced by CA PR OLD was $613.2 \pm 51.5\text{nA}$ compared with $213.5 \pm 14.2\text{nA}$ produced by CA PR2; the older probe produced an almost three-fold higher signal, indicating that the probe redesign had failed to yield an improved product. The CA PR2 probe had been designed to the area 6bp downstream of the start site of the CA PR OLD binding site and extended 4bp beyond it. So the reason for the poor signal produced by CA PR2 compared with CA PR OLD could not be blamed on the choice of target region but must instead be connected with the DNA sequence of the target or the ferrocene label itself. This was not investigated further.

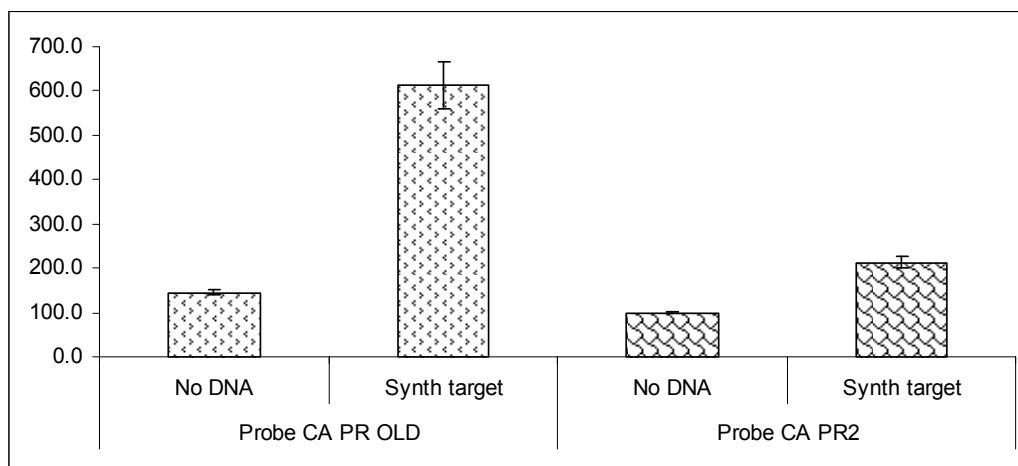


Figure 4.9. Mean currents produced in synthetic assays with CA PR OLD vs. CA PR2. Error bars represent SD (n=3).

Since the previous *C. albicans* specific probes had been designed to a small area of the ITS2 region it was decided to design a new probe, CA PR3, to an entirely different region of the *C. albicans* ITS2 region in an effort to obtain a probe capable generating satisfactory results (figure 4.8). Figure 4.10 shows the results of an assay with target generated from PCR comparing the CA PR3 probe with the CA PR OLD probe. The CA PR3 probe produced mean currents of $76.2 \pm 7.1\text{nA}$ and $80.0 \pm 20.9\text{nA}$ when target DNA was present compared to currents of $46.5 \pm 5.4\text{nA}$ and $41.0 \pm 3.0\text{nA}$ obtained with CA PR. A similar disparity of results was also observed between the currents produced with the DNA-free negative samples: $46.4 \pm 1.5\text{nA}$ and $23.6 \pm 13.9\text{nA}$ for CA PR3 and CA PR OLD respectively. The CA PR3 probe gave a more satisfactory positive signal than CA PR OLD and it was decided to continue with this probe and try and optimize the assay to increase the current produced when target DNA was present.

To achieve this it was decided to readdress the issue of MgCl_2 concentration in the assay. A 1972 paper by Kerr & Sadowski suggested that the optimum conditions for T7 exonuclease activity required a higher concentration than the 1.5mM MgCl_2 that was used in the assay.

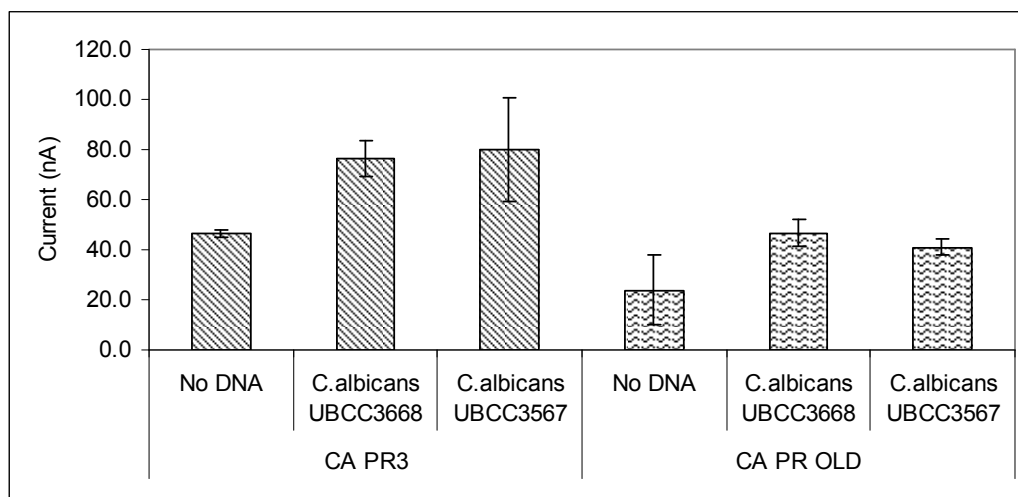


Fig 4.10. Mean currents obtained in electrochemical assays using target obtained by PCR comparing the currents generated by CA PR OLD and the newly designed CA PR3. The error bars represent SD (n=3).

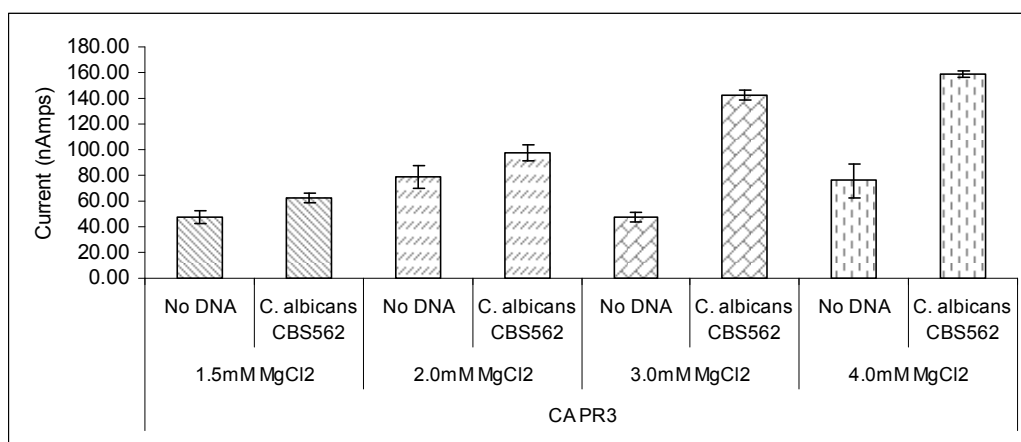


Figure 4.11. Mean currents obtained in experiments testing different amounts of MgCl₂ in the assay with CA PR3. Error bars represent SD (n=3).

previously. Figure 4.11 shows the results from an experiment to test the effect of different MgCl₂ concentrations on the amount of current produced. The results were discussed in more detail in Chapter 3 and so will not be further explored here. According to the above results a MgCl₂ concentration of 3mM gave the best discrimination between background current and the current produced by target-containing sample – $47.9 \pm 3.8\text{nA}$ vs. $142.4 \pm 3.4\text{nA}$. This concentration of MgCl₂ was incorporated into future assays with the CA PR3 probe and these conditions were used to generate the results of the specificity test against nine non-target *Candida* species which can be seen in figure 4.12. The mean current produced when target DNA was present was $162.9 \pm 19.6\text{nA}$ compared with a background current of $50.7 \pm 4.2\text{nA}$ and non-target currents of between $45.1 \pm 1.5\text{nA}$ and $53.8 \pm 6.0\text{nA}$. The differentiation between the signal obtained with target and non-target samples was now sufficient and cross-reactivity was not a problem with any of the species tested. The CA PR3 probe was therefore considered to be satisfactory.

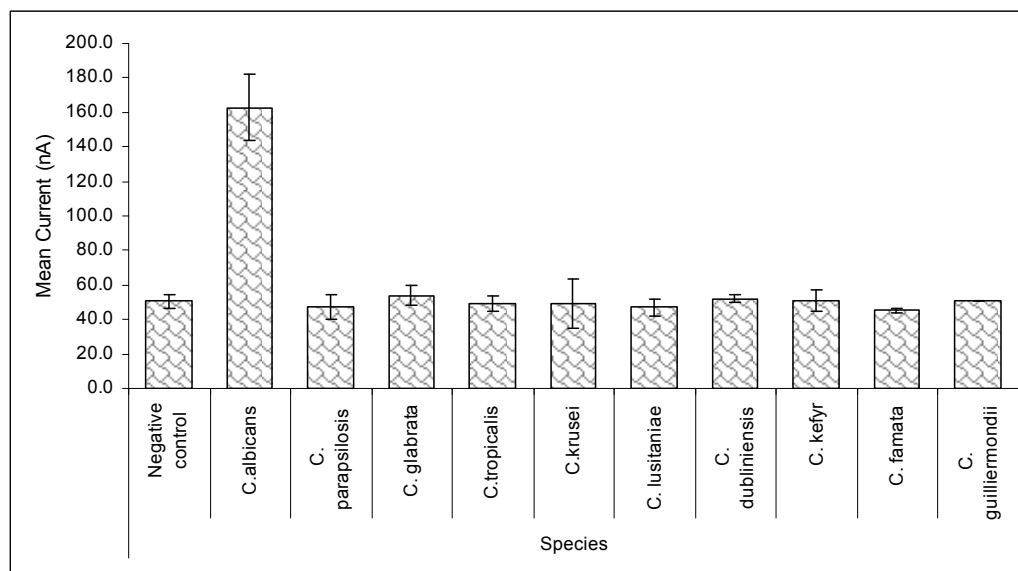


Figure 4.12. Bar graph showing the mean currents produced in assays performed with CA PR3 vs. the test panel of 10 *Candida* species. Error bars represent SD (n=3).

4.3.3 Testing problematic probes: CG PR.

The *C. glabrata* specific probe CG PR was tested for specificity against the panel of 10 *Candida* species and the results are shown in figure 4.13. The mean current produced when *C. glabrata* DNA was present was 120.6 ± 21.6 nA while the mean background current when no-DNA was present was 26.3 ± 7.3 nA. The mean currents produced by non-target species ranged between 21.7 ± 5.0 nA and 98.9 ± 21.3 nA. The results demonstrated that the CG PR probe was cross-reacting with two non-target species, *C. famata* and *C. guilliermondii* which gave mean currents of 94.6 ± 14.4 nA and 98.9 ± 21.3 nA respectively. Analysis of the ITS2 region of these species identified a region of high similarity with the CG PR target site (fig 4.14) which was most likely the reason for the cross-reactivity. The *C. famata* target site had 18 matching bases while the *C. guilliermondii* target site had 17 matches. This discovery underlined the importance of extensive investigation when determining the specificity of molecular assays. Shin *et al.* (1999) performed specificity tests with their species-specific probes using only the other four clinically relevant *Candida* species and so did not screen either *C. famata* or *C. guilliermondii* for cross-reactivity with the *C. glabrata*-specific probe. This was despite the fact that *C. guilliermondii* DNA was available and

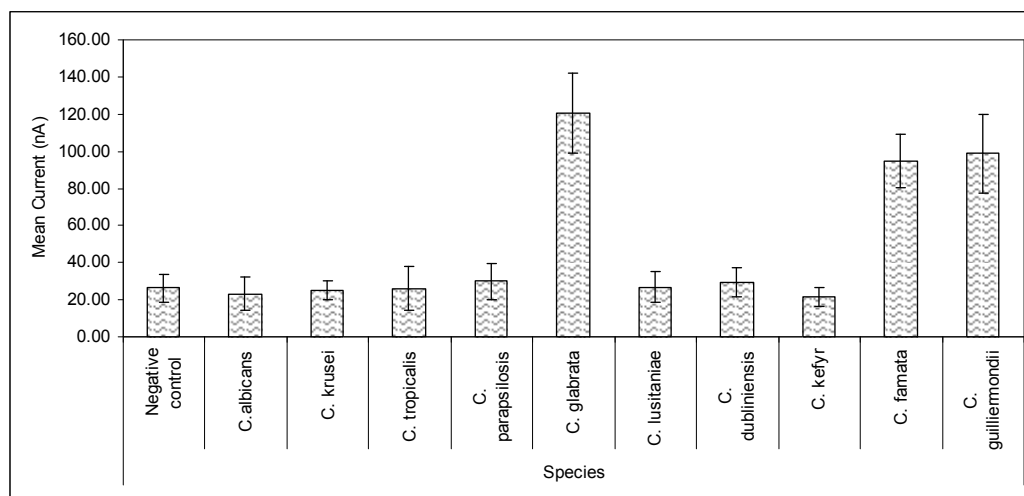


Figure 4.13. Bar graph showing the mean currents produced in assays performed with CG PR vs. the test panel of 10 *Candida* species. Error bars represent SD (n=8).

was used to test the efficiency of their *Candida* genus-specific probe. The result above suggested that Shin's *C. glabrata*-specific probe may not have been specific. However, this was not certain as the procedure used in that method was fundamentally different and used higher probe annealing temperatures which may have preserved the specificity of the probe. Fortunately there were other regions available for probe design in the *C. glabrata* ITS2 region that were sufficiently dissimilar to the other species.

The probe CG PR2 was designed to a different region of the *C. glabrata* ITS2 which was different between the three species. Fig 4.15 shows the mean currents obtained with this probe in a specificity test against the 10 *Candida* species. The mean current produced by CG PR2 with target DNA was 77.0 ± 11.4 nA compared with a mean background current of 26.2 ± 6.7 nA and non-target mean currents of between 26.4 ± 2.5 nA and 44.8 ± 14.6 nA. These results were reasonably good but the signal obtained with target DNA was still quite low compared with the signals obtained with the CT PR and CP PR probes. A possible reason for this emerged when it was discovered that the CG PR2 probe was not an exact match to the *C. glabrata* target site. An error made when transcribing the target sequence from MEGA went unchecked and meant that cytosine was included as the 5' terminal nucleotide when it

should have been guanine. As the 5' nucleotide carries the ferrocene label it was conceivable that a mismatch here would have a substantial negative effect on the signal generated in the assay. As a result it was decided to design another *C. glabrata* specific probe to the same target site as CG PR2 but to extend the probe length by 5bp to make it a similar length to CT PR and CP PR. This probe, CG PR3, was designed in the forward orientation to keep the assay straightforward in terms of primer concentrations in asymmetric PCR. Figure 4.16 shows the results from assays with CG PR3 and the test panel of 10 *Candida* species.

The mean current produced by the probe when *C. glabrata* DNA was present was $116.0 \pm 47.7\text{nA}$ compared to a mean current of $23.7 \pm 4.8\text{nA}$ when no DNA was present and mean currents of between $12.1 \pm 4.4\text{nA}$ and $28.4 \pm 10.1\text{nA}$ when non-target DNA was present. These results were excellent and demonstrated that there was good discrimination between positive and negative samples with no evidence of cross reactivity with any species tested.

4.3.4 Testing problematic probes: CK PR.

The CK PR probe initially gave promising results when first tested (figure 4.17) where it produced a mean current of $127.0 \pm 25.5\text{nA}$ in assays with *C. krusei* DNA compared with a background mean current of $14.9 \pm 5.6\text{nA}$ and mean currents of between $12.6 \pm 5.7\text{nA}$ and $24.0 \pm 3.8\text{nA}$ in assays with non-target *Candida* species. However, as time passed and further work was conducted on the probe it became apparent that the mean current produced by the probe was decreasing. Figure 4.18 shows the mean currents produced by assays with CK PR and *C. krusei* DNA over time and shows a decrease in signal over time with the mean current dropping from $127.0 \pm 25.5\text{nA}$, when the probe was first assayed, to a low of $10.1 \pm 0.8\text{nA}$ after 10 months. The background signal also displayed a decreased signal over this time from $14.9 \pm 5.6\text{nA}$ to $5.1 \pm 1.0\text{nA}$.

C. glabrata	GCATCGATGA	AGAACGCAGC	GAAATGCGAT	ACGTAATGTG	AATTGCAGAA	TTCCGTGAAT	CATCGAATCT	TTGAACGCAC
C. guilliermondiiA....A..T	..T.....
C. famataA....A..T	..T.....
C. glabrata	ATTGCGCCCT	CTGGTATTCC	GGGGGGCATG	CCTGTTTGAG	CGTCATTTCC	TTCTCAAACA	CATTGTGTTT	GGTAGTGAGT
C. guilliermondii	A.A.....T	C.....-	.CCC.G....T....
C. famata	A.A.....T	C.....-	.T.C.G....T....
C. glabrata	GATACTCTCG	TTTTTGAGTT	AACTTGAAAT	TGTAGGCCAT	<u>ATCAGTATGT</u>	<u>GGGACACGAG</u>	<u>CGCAAGCTTC</u>	TCTATTAATC
C. guilliermondiiT-	-----...C	GGAC.-----	---.....---	G.TT.CT..A	AAAGT.TTG.	.ATGG.-----	-----
C. famataT-	-----...C	G.AC.-----	---.....---	G.TT.CT..A	AATGT.TTG.	.ATG..-----	-----
C. glabrata	TGCTGCTCGT	TTGCGCGAGC	GGCGGGGGTT	AATACTGTAT	<u>TAGGTTTTAC</u>	<u>CAACTCGGTG</u>	T-TGATCTAG	GGAGGGATAA
C. guilliermondii	.AG.A..A.A	.A.T..TGT.	.A.-----C.	CTC.A.....AT.T..	AA..G.G.G.	C.G.AT..TT
C. famata	..G.A..G.A	.A.T..T.TA	T.A-----C.	TTC.A.....AT.T..	AA.AG.T..A	T.GTAT..TT
C. glabrata	GTGAGTGTTT	TGTGCGTGCT	GGGCAGACAG	ACGTCTTTAA	GTTTGACCTC	AAATCAGGTA	GGGTTACCCG	CTGAACTTAA
C. guilliermondii	C.-G..A..G	.TG..CC.GC	CTTACA...-	..-CAAAC..AA.....
C. famata	C.CG..A...	.AG..TC.GC	CTTACA.T.T	.A-CAAAC..A.....

Fig 4.14. Sequence alignment of the ITS2 region of *C. glabrata*, *C. famata* and *C. guilliermondii*. The target sites of the *C. glabrata* specific probes are underlined: CG PR; CG PR2; and CG PR3. NB. CG PR2 was designed to target the reverse DNA strand while CG PR3 used the same target sequence as CG PR2, extended by 5bp at the 3' end, but was designed to bind to the forward strand.

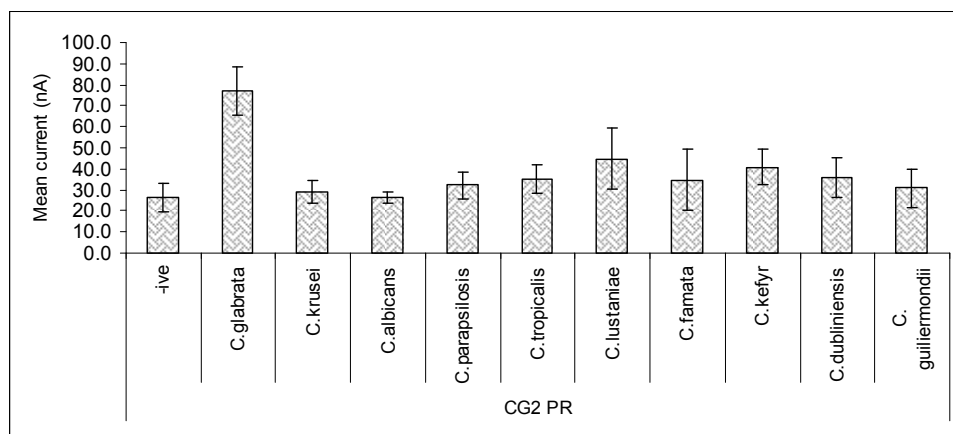


Figure 4.15. Bar graph showing the mean currents produced in assays performed with CG PR2 vs. the test panel of 10 *Candida* species. Error bars represent SD (n=4).

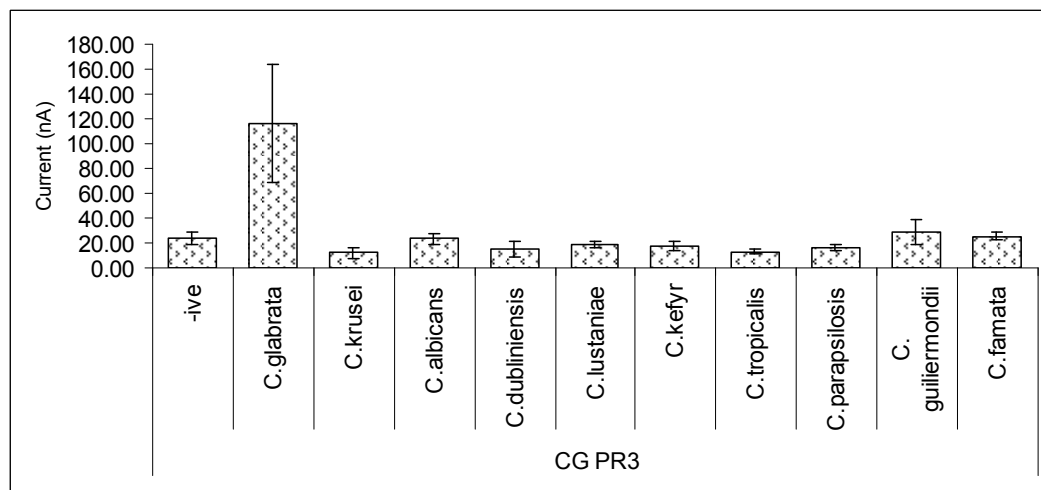


Figure 4.16. Bar graph showing the mean currents produced in assays performed with CG PR3 vs. the test panel of 10 *Candida* species. Error bars represent SD (n=3).

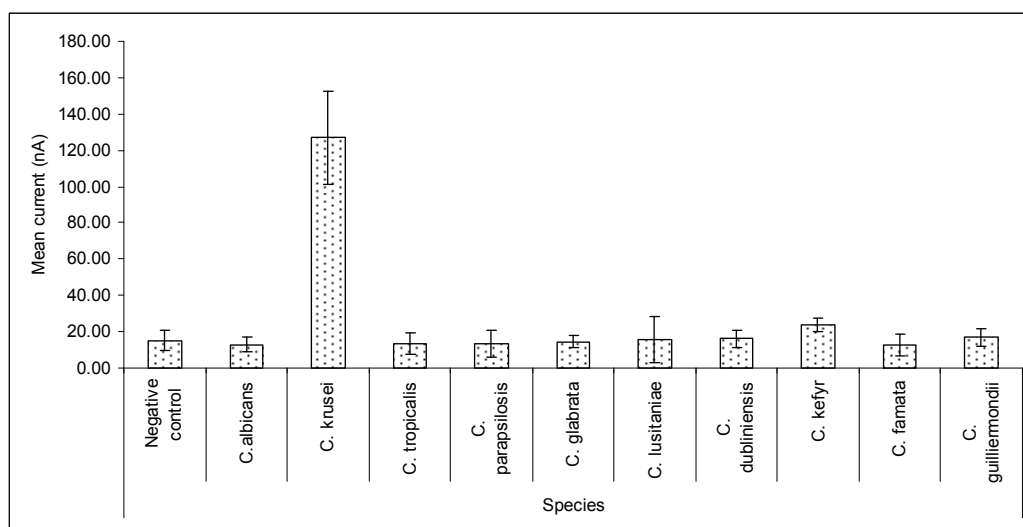


Figure 4.17. Bar graph showing the mean currents produced in assays performed with CK PR vs. the test panel of 10 *Candida* species. Error bars represent SD (n=8).

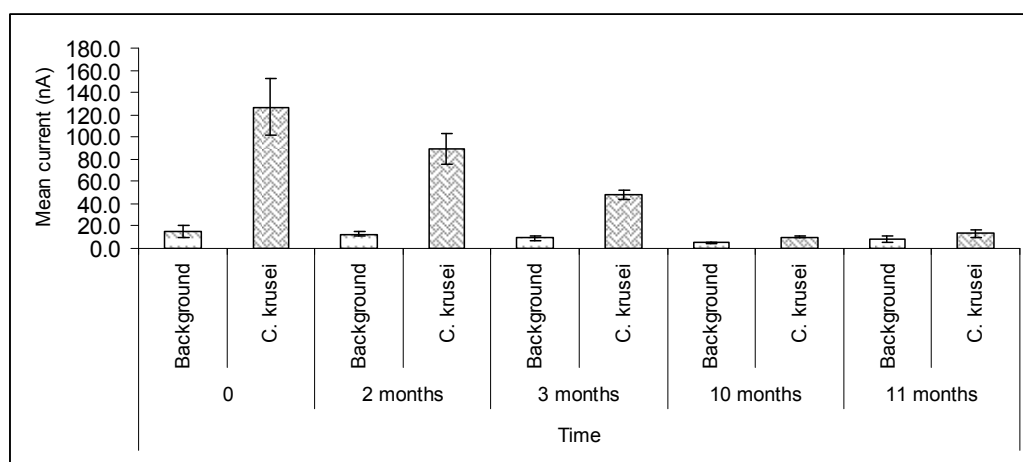


Figure 4.18. Bar graph showing the mean currents produced in assays performed with CK PR using target DNA over a period of 11 months. Error bars represent SD (n=3 for assay at 2 months, 4 for assays at 3, 10 and 11 months, and 8 for assay at 0 months).

The trend was not observed with any of the other probes and was unique to CK PR. Since both the background and positive signals were affected the problem was likely to be related to the probe and/or the label. Upon analysis of the label using the OligoAnalyser program from Integrated DNA Technologies

(<http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx>) the probe was discovered to possess a degree of self-complementarity and may have formed hairpin structures which could have interfered with probe:target binding at lower temperatures (fig 4.19).

If formation of this secondary structure was responsible for the decrease in signal then it may have been possible to restore the signal by denaturing the probe prior to addition to target DNA. Fig 4.20 shows the currents produced in assays using probe that had been denatured for 10s in the presence of target DNA amplified using the Jumpstart PCR conditions.

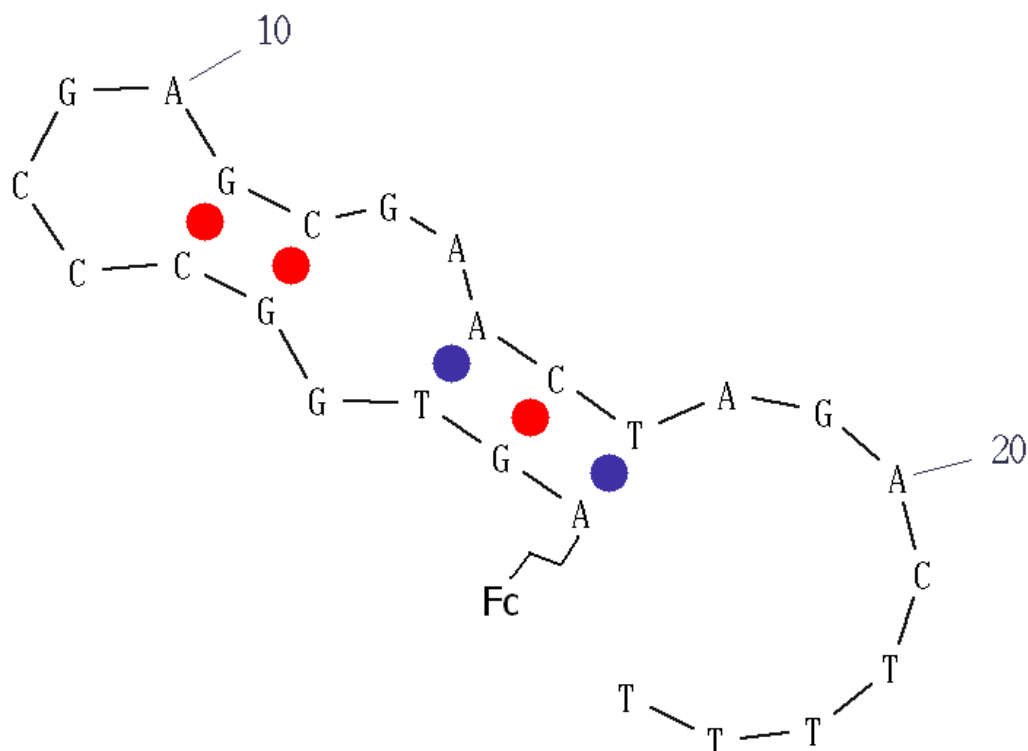


Figure 4.19. Potential hairpin structure of probe CK PR. Fc represents the ferrocene label attached at the 5' end of the oligonucleotide.

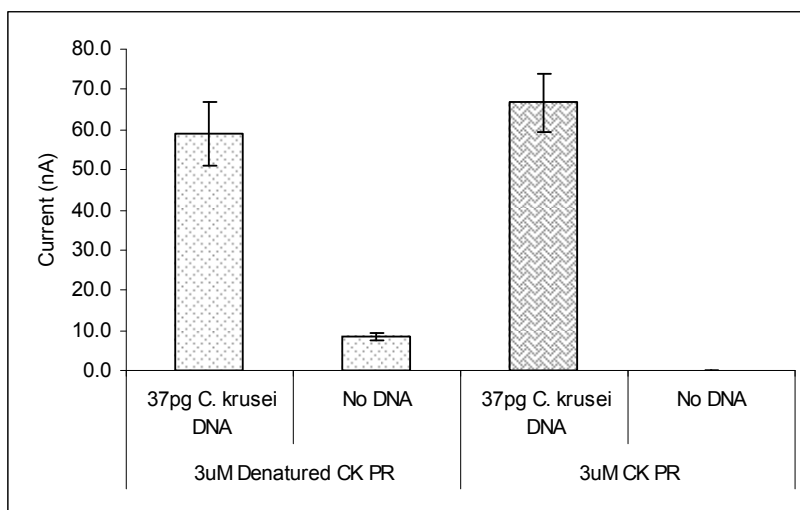


Figure 4.20. Mean currents produced in assays using heat-denatured probe CK PR compared with assays using undenatured CK PR. Error bars represent SD (n=3).

Denaturation did not have a significant effect on the signal produced by the target-containing samples. It did, however, result in an increase in background signal which was probably due to the instability of the ferrocene-oligonucleotide binding at high temperatures as discussed in chapter 7. The decreased signal was not likely to be due to degradation of the ferrocene label as the other probes had been kept under the same conditions as CK PR and had not displayed any loss of signal. Furthermore this decline in signal could not be explained by degradation of the probe oligonucleotide as this would have resulted in an increase in the background signal as shorter ferrocene-labelled nucleotide fragments would be present in assays and yield higher currents.

Another potentially problematic issue with CK PR was the presence of two 'indel' events in the probe relative to the target sequence. Fig 4.21 shows the alignment of CK PR to its target site where it can be seen that there is a missing cytosine at position 3 which causes the sequence to misalign for 4bp before inclusion of an extra cytosine at position 6 which brings the remainder of the sequence back into alignment. Had the probes been designed *de novo* this error would not have occurred but the probe sequences were taken

```

CK PR:      5' agtggcccgagcgaactagactttt 3'
            || | | | | | | | | | | | | | | | |
Target:     5' agctggccgagcgaactagactttt 3'

```

Figure 4.21. Alignment between probe CK PR and its target site in *C. krusei* ITS2 region.

directly from the paper by Shin et al. and were assumed to be flawless. While it is a distinct possibility that this pattern of mismatches so close to the crucial ferrocene-labelled 5' end of the probe would have a detrimental effect on binding and therefore exonuclease digestion and current production, it doesn't explain why the initial results with CK PR produced satisfactory currents which then decreased over a period of months to levels which were approximately a tenth of the original values.

The exact cause of the decreasing current levels produced by CK PR was never discovered but there were obvious problems with its design that would be expected to be detrimental to its performance. While a new probe could have been designed with relatively little difficulty in the ITS2 region, the expense of probe synthesis and ferrocene coupling combined with the risk that a new probe may not have been satisfactory meant that this was never attempted. The currents with CK PR, while much lower than the other probes, were still able to discriminate between background current and current from a target-containing sample and were deemed adequate for continued investigation.

4.3.5 Testing probes using DNA from clinical *Candida* isolates

All of the above work used the type strain of each *Candida* species but to ensure that the probes could detect wild isolates it was necessary to test the assay using DNA from clinical strains of *Candida* species. Assays were performed using DNA from four wild isolates of the relevant species as well as from the type strain and DNA-free samples were also included to produce background values for each probe.

All species-specific probes were able to detect DNA from wild isolates (Fig. 4.22). The currents obtained with the wild isolates were of similar values to the currents generated by the type strain of each species, despite the wide range of geographical and/or environmental origin of the wild isolates, indicating that there were no stain-related differences within these species and the probes were able to reliably detect them, although the number of isolates tested was relatively small due to the limited availability of strains. There was some variation between the signals obtained by different probes with CK PR giving the lowest signals (as would be expected) of around 40nA and CA PR3 and CP PR giving the highest of around 140nA.

These results demonstrated the ability of the probes to reliably detect strains that were not just wild isolates but had been isolated from patients with candidaemia.

4.4 Testing PanF PR probe against fungal isolates

The results of testing detection using extracted DNA from 15 fungal species are shown in figure 4.23. The mean currents obtained varied relatively widely from $118.2 \pm 15.4\text{nA}$ (*Cr. victoriae*) to $385.4 \pm 35.5\text{nA}$ (*Cr. gattii*) compared to the background signal of $29.4 \pm 4.4\text{nA}$. This demonstrated that DNA from a wide variety of fungi could be successfully amplified and detected using the PanF PR probe and associated primers, and was confirmation of the utility of the developed bioinformatic approach of probe design. The assay was a success and the currents produced were generally higher than seen before. The currents produced ranged widely between species but the exact reason for this was not known. One possible reason was that differences in the amount of template DNA added to the PCR caused a wide variation in the signal produced, as the amount used was never standardized. Out of the 15 species tested, 14 are associated with human disease; *Cr. victoriae* is a known plant pathogen but no cases of its causing human disease have been reported.

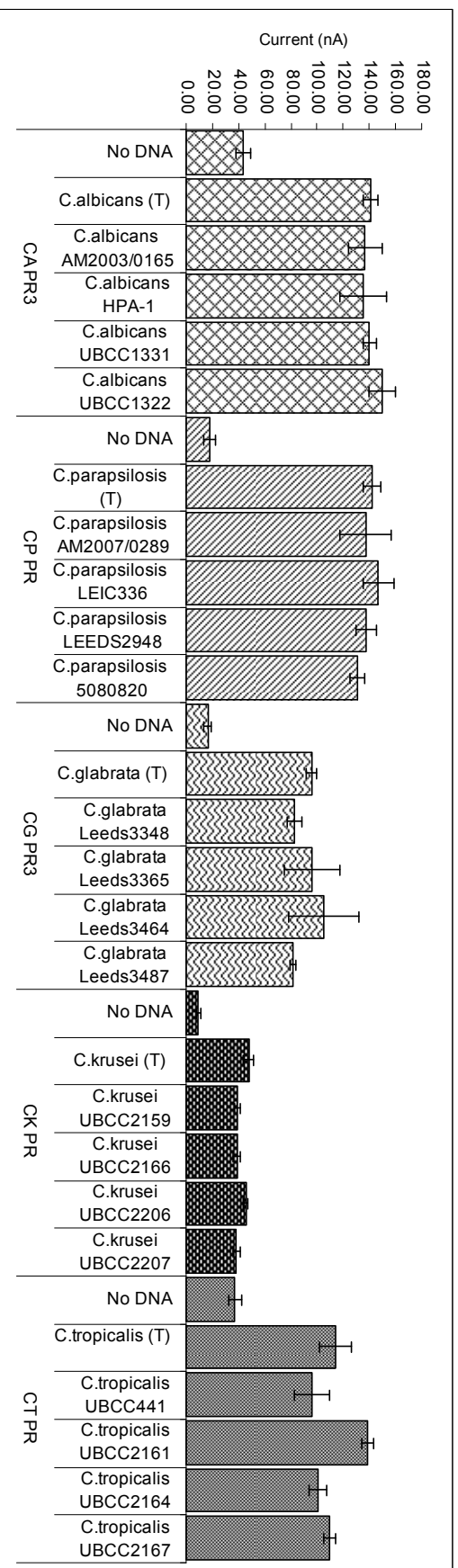


Figure 4.22. Mean currents produced by assays with all 5 *Candida*-specific probes to detect DNA from wild isolates of the relevant species. Error bars represent SD (n=4).

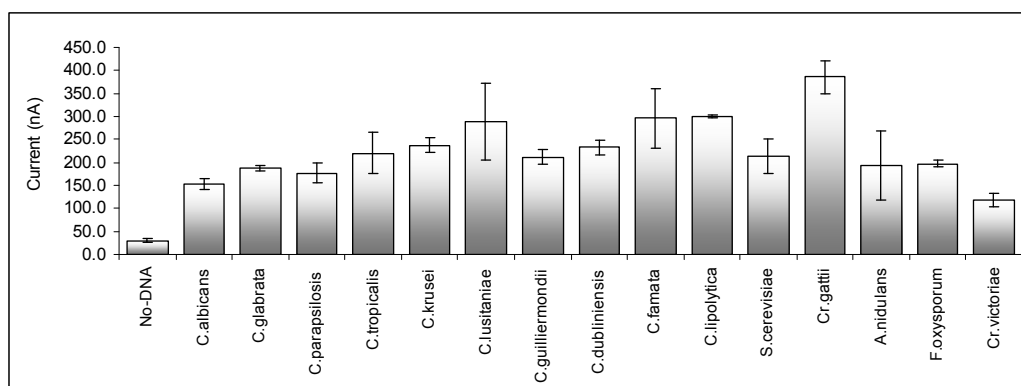


Fig. 4.23. Mean currents produced by assays using a pan-fungal probe to detect DNA from 15 fungal species. Error bars represent standard deviation (n=3). These results show presence of fungal DNA in a sample.

4.5. Testing the probes against human DNA

Each of the probes was tested against fungal target DNA in the presence of sufficient human DNA to provide approximately the same number of rDNA copies as the fungal target. The mean currents produced by PanF PR and CK PR, which were the best and worst performing probes respectively, are shown in Fig. 4.24. Successful discrimination was achieved with all probes and the presence of human DNA alone did not increase the signal above the background, indicating that cross-reactivity was not an issue. The currents produced by the mixed fungal+human DNA samples also indicated that sequestration of primers by human DNA was also not an issue when this amount of DNA was used.

While equal numbers of human rRNA and fungal rRNA targets would not arise in a clinical sample in which the ratio of human cells to fungal cells is extremely high, of the order of $10^9:1$ it was not possible to test such a condition with the assay in its current form. This more clinically realistic situation was tested in chapter 6 where attempts were made to detect fungal DNA in spiked blood samples.

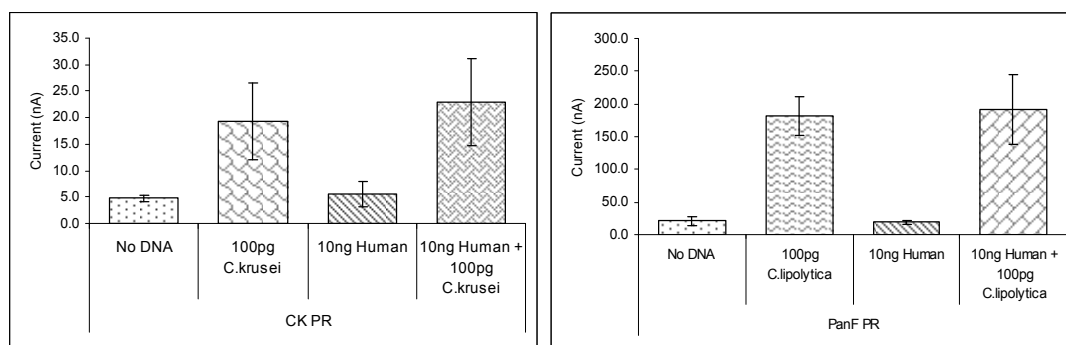


Figure 4.24. Mean currents produced in assays with probes CK PR and PanF PR to test the effect of human DNA on the assay. Error bars represent SD (n=3).

4.6 Conclusions.

The results showed that the *Candida* species-specific probe suite consisting of CA PR3, CP PR, CG PR3, CT PR and CK PR are specific to their target species and are capable of detecting DNA from clinical isolates as well as the type strain. There were some difficulties encountered with the design of probes – from problems with their specificity (CG PR) to an inexplicable degradation of signal over time (CK PR). The former problem was able to be overcome by probe redesign but the latter problem was not immediately apparent and as a result was not formally investigated due to time and cost constraints.

In the process of redesigning the probes a useful method was developed using bioinformatics analyses which could predict cross reactivity between probes and non-target species and could be used to screen potential probe sequences, greatly speeding up the process of probe design and optimization. This method led to the design of the successful panfungal PanF PR probe which gave relatively high signals with 15 fungal species tested.

The probe suite was now decided upon and the specificity of the probes confirmed. In order to be useful the probes had to be shown to be able to detect DNA from very low amounts of *Candida* DNA. The next step was to optimize the assay to enable detection of a single genome of fungal DNA in an assay with all of the probes.

Chapter 5 - Limit of Detection

5.1 Introduction

Invasive fungal infections (IFI) can be diagnosed definitively upon isolation and identification of fungi from blood but diagnosis is often made based on suspicion if fungi are recovered from other normally sterile sites, such as CSF, and if the patient exhibits numerous risk factors that predispose towards IFI. These include a weakened immune system, recent surgery to the gastro-intestinal tract and failure to respond to antibiotics. The diagnosis of IFI on clinical suspicion is of particular importance as conventional diagnosis relies on culture of fungi from a clinical sample and this takes days to achieve; furthermore the sensitivity of culture has been questioned and a fungus may not be recovered at all. With this in mind, diagnosis on clinical suspicion alone allows antifungal treatment to be administered immediately and increases the likelihood of patient recovery. There are still problems inherent in this approach, however, and the main issue is that of antifungal drug resistance. There are numerous antifungal agents that may be administered to treat IFIs but not all of them are effective against all species of fungi and their use must be directed both by the cost of treatment and the production of possible side-effects in the patient. Since clinical suspicion alone cannot reliably identify the species of fungi which is causing an IFI a clinician must await the results of further tests to ascertain the identity of the offending organism. Molecular methods based on PCR offer rapid identification of fungi in clinical samples to species level by using unique stretches of the genetic code to allow detection and identification of fungi. These tests are much more rapid than culture and have the potential to greatly increase the accuracy of diagnosis of IFIs, provided they are capable of detection of clinically relevant amounts of fungal cells in clinical samples.

Of the different types of clinical sample that can be tested to try and detect fungal DNA blood is the most likely sample type to use in the case of a disseminated fungal infection leading to IFI. It is a potentially difficult sample type to deal with as there is a very large background of human cells present as well as a large number of macromolecules which would interfere with

molecular processes such as PCR and detection by fluorescence methods. Any method involving amplification of fungal DNA in blood would require pre-treatment to remove this material prior to nucleic acid amplification. This could be achieved by capturing and separating out the fungal cells from blood and using them directly in a PCR reaction, with without first extracting total DNA. The other option is to simply extract total DNA from the blood sample so that the mix of extracted fungal and human DNA is used in the downstream PCR reaction. The latter method is considerably easier to achieve but relies on specificity of the primers and probes for target fungal DNA, otherwise there is the possibility of false positives and false negatives being produced, particularly given the very small amounts of fungal DNA present in the samples compared with human DNA. With this in mind it is easy to see why it is of paramount importance that the PCR conditions are optimised for amplification of very low amounts of target DNA. Key factors to achieve this are use of a hotstart *Taq* DNA polymerase to prevent amplification of undesired products in the initial stages of PCR, as well as primers which have been designed to minimise interactions at their 3' termini.

The symptoms of fungaemia may present with a fungal load as low as 5-10 c.f.u. ml⁻¹ of blood and may be over 100 c.f.u ml⁻¹ (Loeffler *et al.*, 2000) so it is essential that the fungal detection assay be capable of detecting fungi in this range. Following the successful test of the specificity of the *Candida* species-specific probes the next step was to test the sensitivity of the assay and, if necessary, optimise the procedure to allow detection of a single cell of *Candida* yeast in a 1ml blood sample. Before attempting to detect cells in a blood sample it had first to be confirmed that the assay was capable of detecting extracted DNA equivalent to a low number of fungal genomes.

Genome size data from the online fungal C-values database (Kullman *et al.*, 2005) was used to determine the mass of a single genome of each of the *Candida* species in order to prepare appropriate dilutions of extracted genomic DNA. The only estimates available for a diploid genome were for *C. albicans* which reportedly had a 37fg diploid genome and it was decided to use this value for all of the diploid *Candida* species (*C. albicans*, *C.*

parapsilosis, *C. tropicalis* and *C. krusei*); the estimate used for the genome size of the haploid *C. glabrata* was 13fg. Appropriate dilutions of extracted DNA were made based on these estimates.

5.2 Initial attempt at detection of low amounts of extracted DNA

The first experiments into assay sensitivity used the improved PCR conditions that were outlined in section 2.7.3 with the exception that the original primers ITS3 and ITS4 were used. DNA dilutions corresponding to the diploid C-value were not made in the early stages of experimentation, hence the amounts of DNA used do not correspond to 1, 10, 100 etc. genomes.

Figure 5.1 shows sample results from assays attempting to detect between 50pg and 15fg of DNA (equivalent to ~1300 genomes and ~0.41 genomes) with probes CT PR and CP PR. The assay with CT PR was only able to detect down to 500fg of *C. tropicalis* extracted DNA while the assay with CP PR could detect reliably down to 500fg DNA but produced inconsistent detection at 50fg and 15fg with only one assay out of three producing a signal; the DNA-free sample also produced a very low signal in one out of the three replicates. The results demonstrated that the new conditions of increased MgCl₂ concentration and use of a hotstart *Taq* polymerase were not sufficient to allow detection of the smallest amounts of *Candida* DNA.

The PCR reaction is the limiting step of the assay because if insufficient target amplicon is produced it leads to little or no current being generated in the following probe hybridisation-digestion reaction. Therefore optimisation of the PCR was deemed to be the most important step to permit detection of 1 genome of *Candida* DNA.

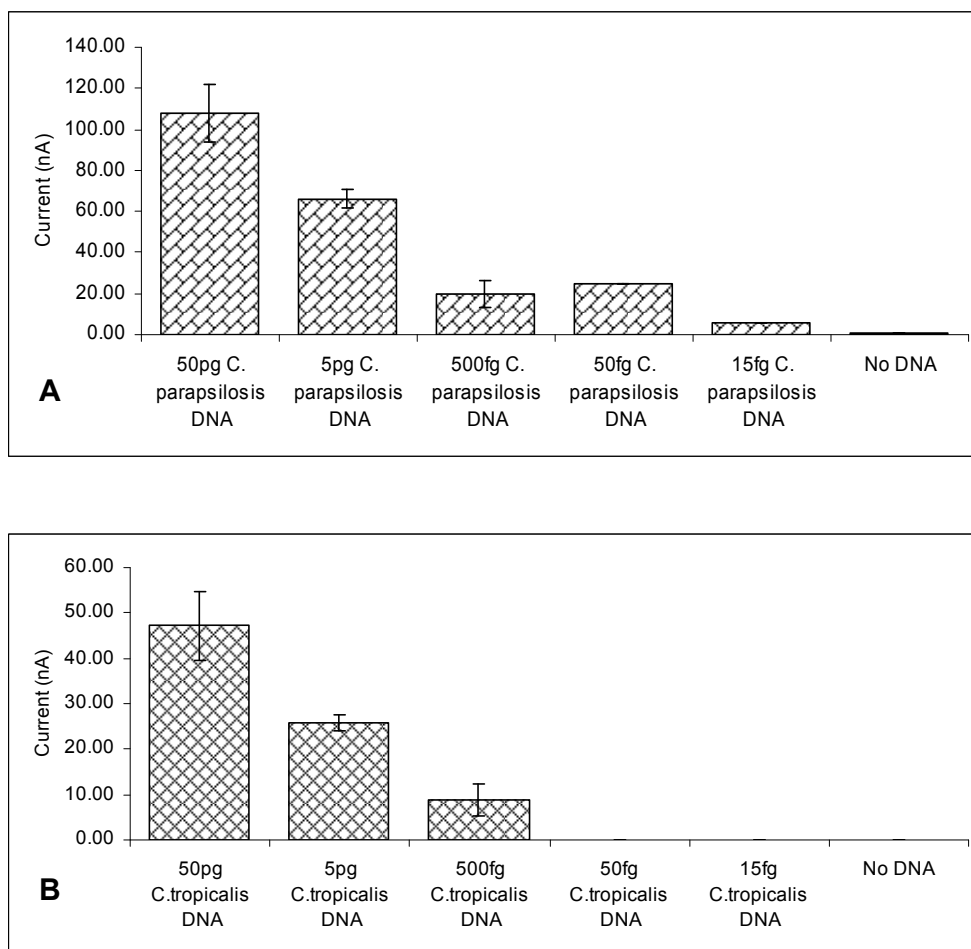


Figure 5.1. Mean currents obtained with low amounts of (A) *C. parapsilosis* and (B) *C. tropicalis* genomic DNA. Error bars represent SD (n=3).

5.2.1 Further optimisation of PCR conditions for detection of low amounts of *Candida* DNA – cycling conditions.

There were several aspects of PCR that had by now been optimised to achieve low levels of detection: $MgCl_2$ concentration, *Taq* polymerase concentration and *Taq* polymerase properties (i.e. hotstart DNA polymerase). The cycling time had never been examined before and it was reasonable to hypothesise that the straightforward step of increasing the number of cycles could increased amplification of target DNA. An assay was carried out using *C. tropicalis* DNA to compare the signal obtained when PCR was carried out at 50 cycles, compared with the signal produced following a 40 cycle PCR (fig 5.2).

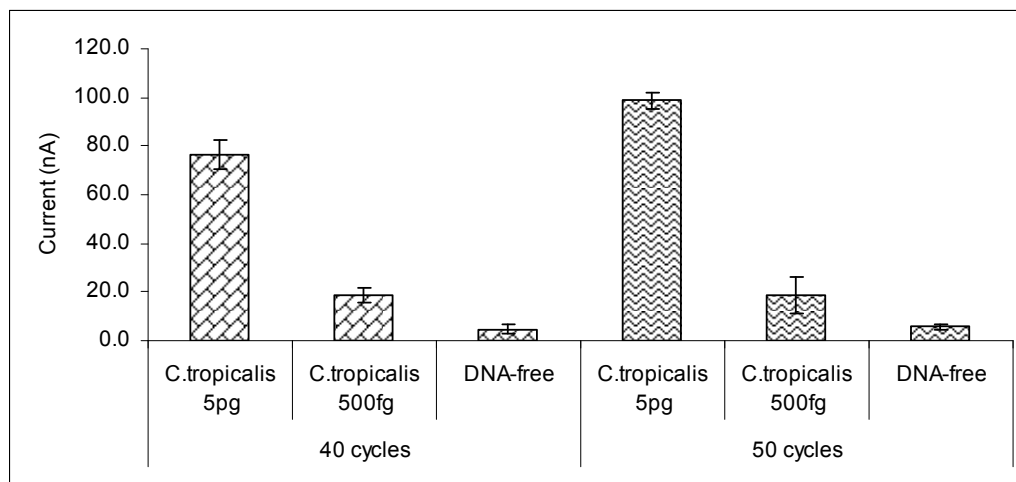


Figure 5.2. Mean currents produced in an assay using *C. tropicalis* DNA amplified by 40 cycle PCR compared with 50 cycle PCR. Error bars represent SD (n=3).

The mean current produced with 5pg DNA at 50 cycles was significantly higher than the signal produced at 40 cycles, $98.8 \pm 3.2\text{nA}$ vs. $76.6 \pm 6.1\text{nA}$ respectively ($P < 0.005$; t-test, $n=3$). However, while the $\sim 20\text{nA}$ difference in signal was statistically significant, it was not a very large difference in actual terms and similar fluctuations in mean current had been observed in results from assays carried out using identical conditions at different times. Furthermore, the mean current obtained using 500fg of DNA at 40 cycles was actually slightly higher than it was at 50 cycles, $19.0 \pm 3.0\text{nA}$ compared to $18.5 \pm 7.4\text{nA}$. These results demonstrated that simply increasing the number of cycles of the PCR was not sufficient to increase the current produced by the assay and therefore allow reliable detection of low amounts of fungal DNA.

5.2.2 Further optimisation of PCR conditions for detection of low amounts of *Candida* DNA – primers.

Primer sequence and concentration can affect the degree of amplification of target DNA in PCR. Ultimately, the amount of target that can be amplified is related to the amount of primer molecules available to the reaction. However, the relationship between amount of primer and amount of product is unlikely to remain linear; Li *et al.* (1990) suggested that primer-dimerisation and mis-priming occur when PCR is carried out for >35 cycles

and the presence of larger amounts of primer would increase the probability of these events occurring. This would produce alternative targets for primer annealing and so decrease the availability of primers in the reaction and decrease the yield of the target amplicon, particularly when a low amount of starting template is present.

A reasonable assumption to make was that an optimal, increased concentration of ITS3/ITS4 primers existed which would allow a greater amount of target DNA to be amplified during PCR which would result in a higher signal being produced following probe hybridisation-digestion. Since the PCR conditions are designed for asymmetric amplification of DNA it was feasible that increasing the amount of the primer which amplified the target strand would result in increased ssDNA target and therefore a higher current produced by the assay. To test this hypothesis ratios of 10:1 and 20:1 of target-producing primer:non-target producing primer (ITS4:ITS3) were used in assays with *C. tropicalis* DNA to compare the signals obtained with the standard 5:1 primer ratio. The results (shown in fig 5.3) demonstrated that there was no significant difference between the signals produced when the amount of ITS4 used in the reaction was varied, indicating that the 5:1 ratio of ITS4:ITS3 was not limiting the signal produced by the assay and therefore was not limiting the amount of product amplified by PCR.

It was still possible that changing the primer concentrations could yield more target. Whilst it was the excess ITS4 in the PCR which produced the ssDNA target for the probes, a suitable concentration of ITS3 was crucial in the early, exponential cycles of the PCR in order to produce enough template for the later, linear cycles of PCR which would yield ssDNA target. By proportionally increasing the amount of each primer in the reaction it was possible that an increase in the amount of target would be achieved and therefore an increased production of current. Fig 5.4 shows the mean currents produced in assays using five-fold higher concentrations of the ITS3 and ITS4 primers, 500nM and 2.5µM respectively compared with the usual

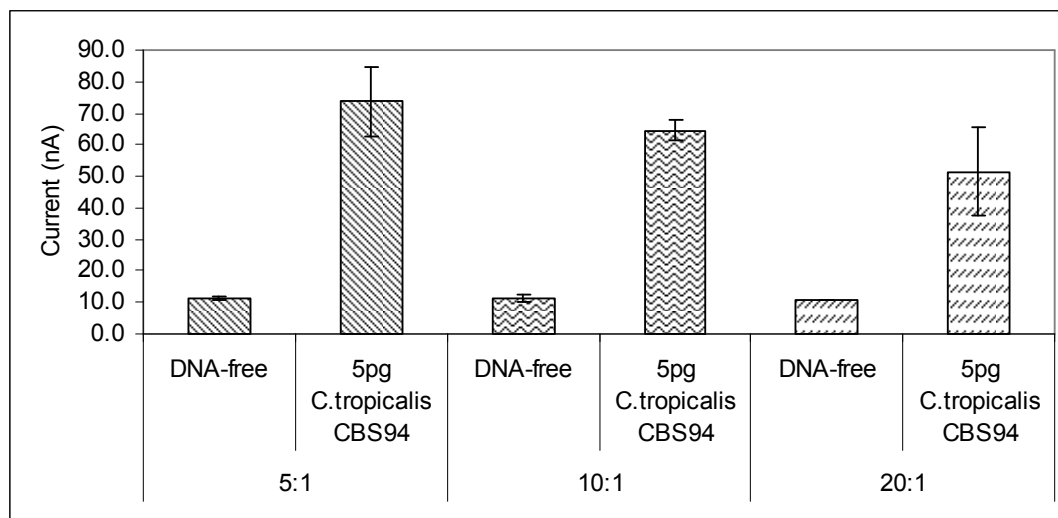


Figure 5.3. Mean currents produced by assays with target DNA amplified by different ratios of ITS4:ITS3 primers using *C. tropicalis* DNA. The error bars represent SD (n=3).

conditions of 100nM ITS3 and 500nM ITS4. These results showed a highly significant decrease in signal when the primer concentrations were increased – the mean current decreased from $59.9 \pm 5.6\text{nA}$ to $11.3 \pm 2.1\text{nA}$ when five-fold greater concentrations of the primers were used ($P < 0.0001$).

This decrease in signal was very likely due to the primer interactions mentioned by Li *et al.* occurring at an early stage of the PCR due to the large excess of primer molecules present in the reaction. Such interactions and consequent formation of primer-dimers would also have occurred in reactions in which the primers were present at their ‘standard’ concentrations. However, at these lower concentrations they would not have become such a dominant species of amplified material in the early stages of the PCR reaction and the genomic DNA target would have provided adequate template to produce the desired target DNA.

To demonstrate that this was likely to be the case, some degree of interaction would have to be identified in the primers. The Oligoanalyser program provided by Integrated DNA Technologies was used to analyse the primers for possible interactions leading to primer artefacts. It was discovered that the primers had complementary 3' ends consisting of two G-C bonds (fig 5.5) and

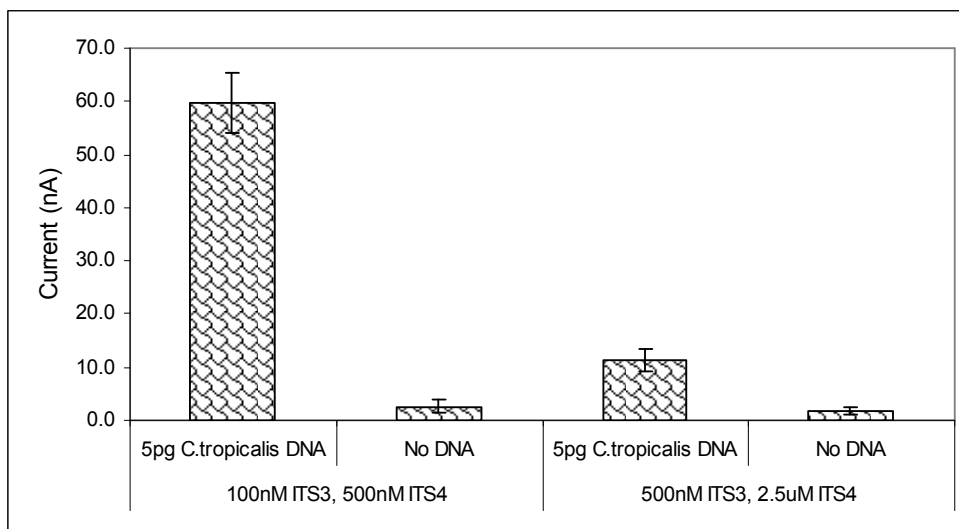


Figure 5.4. Mean currents produced in assays with *C. tropicalis* DNA amplified with two different concentrations of the primers. Error bars represent SD (n=3).

this was thought to have contributed to the decreased signal observed when the concentration of the primers was increased. Due to this potential defect it was decided to design new primers for amplification of the ITS2 region in fungi that were free from such 3' interactions that could limit amplification of target DNA.

Following the design and testing in pair-wise combinations of three new forward and three new reverse primers designed to the 3' end of the 5.8S rRNA gene and 5' end of the 26S rRNA gene respectively, the primer pair ITS3.3 and ITS4.2 was selected as the most efficient primer pair since they could amplify DNA equivalent to ~1.08 genomes of *C. tropicalis* to levels that were visible on an agarose gel (fig 5.6). There was evidence of contamination in the samples amplified using ITS3.1/ITS4.2 and ITS3.3/ITS4.2, suggesting that the ITS2 primer was contaminating.

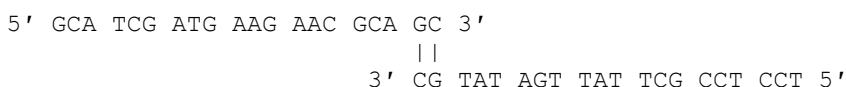


Figure 5.5. Alignment showing the complementary 3' ends of primers ITS3 (top) and ITS4 (bottom).

Unfortunately this was not realised at the time – the contamination observed was assumed to have occurred by chance in this experiment and the possibility of contamination of the stock ITS4.2 primer was not considered. This possibility may have led to the difficulties experienced later on when detection of low amounts of DNA was attempted (see below and chapter 6). Nevertheless, the primers were then tested in electrochemical assays to detect 4pg and 40fg of DNA extracted from *C. parapsilosis* (equivalent to ~108 and ~1.08 genomes). The mean currents that were produced are presented in figure 5.7 and were much higher than seen before: when 4pg DNA was used as target the mean current produced was $330.0 \pm 30.4\text{nA}$ and when 40fg DNA was amplified by ITS3.3/4.2 the mean current was $118.8 \pm 49.2\text{nA}$. This result demonstrated a great improvement in production of electrochemical signal with the assay and showed just how important the PCR step was for the production of current and, in particular, how important it was to have efficient primers for amplification of small amounts of starting template DNA.

The successful detection of 40fg of *C. parapsilosis* extracted DNA (equivalent to ~1.08 genomes) was a great leap forwards towards reliable detection of a single genome of DNA in a reaction which was essential to detection of a single genome in a 1ml sample. However, this result also demonstrated that PCR amplification of 40fg of target DNA was not completely reliable as only two out of three replicates gave a PCR product and concurrent signal and the currents produced were quite different, 153.6nA and 84.0nA.

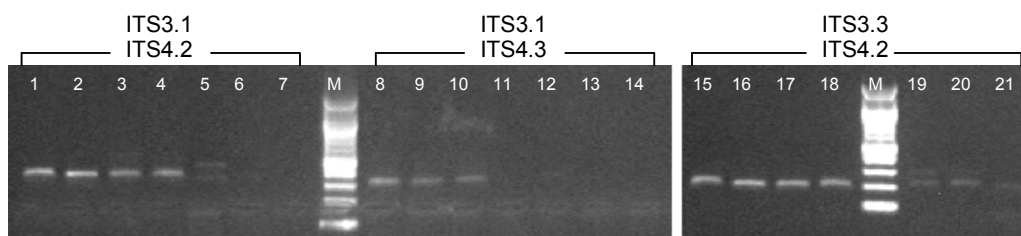


Figure 5.6. Gel image showing PCR products obtained in reactions with three improved primer pairs using low amounts of *C. tropicalis* genomic DNA as template: M – 100bp ladder, 1-2, 8-9 & 15-16 – ~100 genomes *C. tropicalis* DNA, 3-4, 10-11 & 17-18 – ~10 genomes *C. tropicalis* DNA, 5-6, 12-13 & 19-20 – ~1 genome *C. tropicalis* DNA, 7, 14 & 21 – DNA-free negative control.

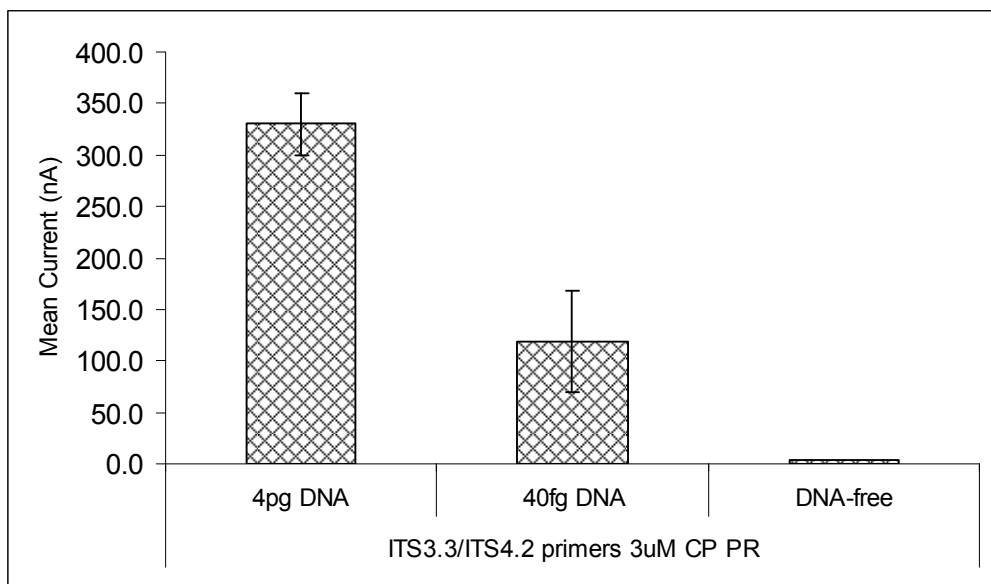


Figure 5.7. Mean currents produced in assays with *C. parapsilosis* DNA amplified by ITS3/ITS4 primers compared with ITS3.3/ITS4.2 primers. Error bars represent SD (n=3 for 4pg DNA and DNA-free results and n=2 for 40fg result as one replicate failed to give PCR product).

This result led to the routine set up of 6 replicates of PCR amplifications with ~1 genome equivalent of DNA added to try and obtain at least 3 data points with which to calculate a mean and standard deviation. This would not validate the assay in terms of reliability of detection of 1 genome of DNA in a reaction but would simply allow the determination of the levels of signal obtained by each probe to show that detection was possible. The question of reliability of the assay could then be addressed once detection of a single genome could be shown to be obtainable by all of the probes.

5.3 Contamination.

The generic nature of the primers used to amplify the ITS2 region and 18s rRNA region was advantageous as it greatly simplified the setup of the PCR reaction to generate target DNA for the assay and would allow for the addition of new probes for the detection of other fungal species to the assay with minimal optimisation but it was not without potential problems. An obvious issue is the situation where DNA from one or more other fungal species was present alongside DNA from the target species. These species would then be in competition for PCR reagents which would likely cause a

reduction in the amount of target DNA, leading to lower currents and an increased risk of a false negative being reported.

Another possible problem was the production of false positives due to contamination by target fungal nucleic acid present in the environment. One possible source of this was in the form of fungal cells present in the environment. The use of gloves when preparing reagent mixes etc. would be enough to prevent commensal species such as *C. parapsilosis* and *C. albicans* from contaminating PCR mixes, but the presence of airborne cells delivered by the air conditioning apparatus could not be ruled out. This was a particular issue due to the location of the lab - beside a wooded area with a stagnant body of water and a deal of decomposing vegetation which would provide an excellent habitat for many species of fungi including *Candida*. A more troublesome source of contaminating DNA was cell-free target DNA amplicon which may have been released following handling of PCR products e.g. when loading agarose gels for analysis. This source of contamination would only become evident over time as levels of amplicon built up over successive rounds of post-PCR sample handling.

False positive and false negative results caused by contamination had not been observed when testing the probes for specificity. There were a number of reasons why this was the case. Firstly, the use of large amounts of target DNA (10 – 100ng) in the PCR reactions meant that there would have been little competition for PCR reagents and so false negative results would not have been observed. Secondly, as discussed above, the ITS3/ITS4 primers used in these test were not sensitive enough to detect small amounts of target DNA hence a small amount of contaminating material would have gone unnoticed. Thirdly, fewer PCR amplifications had been carried out and so there was less contaminating amplicon present in the atmosphere. After performing PCR amplifications for some time the products began to exhibit contamination, so to address this PCR reagents were stored and PCR master mixes were prepared in a 'DNA free' room where amplified DNA was never admitted. However, even under these conditions extensive contamination was observed in the PCR products – a comparison between reactions set up in the 'DNA free' room and in a class 2 biological safety cabinet (BSC)

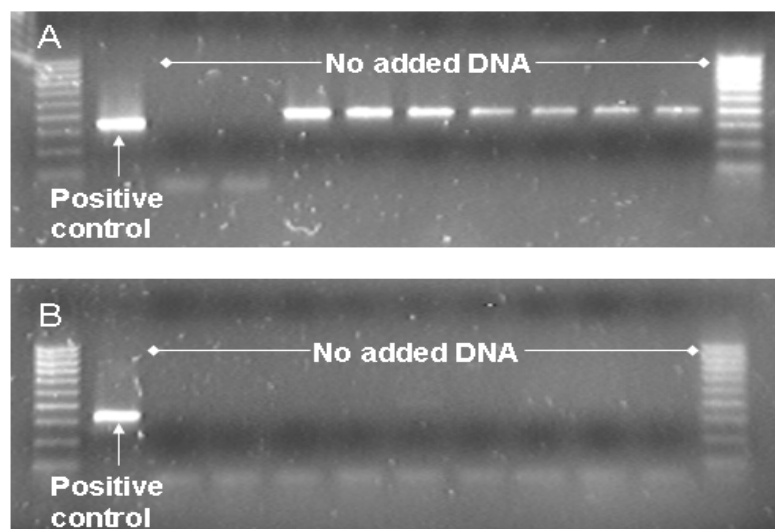


Figure 5.8. DNA-free PCR products set up in (A) 'DNA-free' room and (B) class 2 BSC.

demonstrated that the 'DNA free' room was quite heavily contaminated by fungal nucleic acid (fig 5.8) which was likely to have been delivered by the air conditioning unit installed in the room. The uniform size of the amplicon also suggested a single source of contaminant nucleic acid. As a result of this test, set up of PCR mixes was moved to the class 2 BSC. With the additional provision of an UV source and use of a pre-prepared dNTP mix (Promega) contamination was limited, although it was never eliminated altogether - fig 5.8 shows examples of PCR products obtained in different assays, following the move to the safety cabinet, which show very different instances of contamination.

The varying amplicon size of contaminating material in the reactions with 37fg of *C. parapsilosis* DNA suggested that the contaminating material had come from several different sources. This suggested that it was unlikely that one or more of the PCR reagents had been contaminated, as contaminating amplicons would then be uniform in DNA-free samples, and the use of the class 2 BSC meant that environmental sources of contaminating DNA could be ruled out. As a result, it was thought that the contaminating material in this case originated from amplified DNA present in the atmosphere. As shown in figure 5.9 the contamination of samples was sporadic and so, in order to achieve sensible results demonstrating limit of detection of 1 genome of DNA *in vitro*, it had to be shown that an electrochemically analysed PCR sample

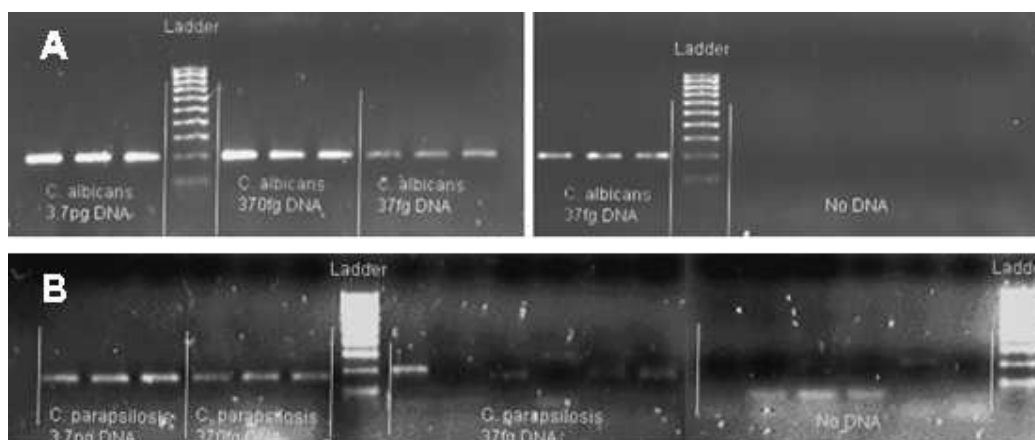


Figure 5.9. PCR products obtained with ITS3.3/ITS4.2 primers when attempting to amplify a range of (A) *C. albicans* DNA and (B) *C. parapsilosis* DNA. Note the range of sizes of contaminating bands in image (B).

which produced no current either contained no DNA or contained non-target DNA, either of which could be regarded as a negative sample for the purposes of the assay.

5.4 Demonstration that no signal indicates no target.

Since the problem of contamination could not be completely overcome it was essential to show that a result with zero current corresponded either to a PCR product with no amplicon or to a non-target contaminating amplicon. An opportunity to demonstrate the latter point arose when performing an assay with the CG PR3 probe. The DNA-free negative samples all showed signs of contamination with bands of several different sizes present. However, the region of *C. glabrata* amplified by primers ITS3.3/ITS4.2 is easily distinguishable from the other *Candida* species due to its size of 274bp. Fig 5.10 shows the products from the PCR amplification with DNA-free samples in this experiment. The samples in lane 2 and lane 6 appear to be the correct size to be product from *C. glabrata* DNA and they were also the only DNA-free negative samples to produce a current when analysed electrochemically with probe CG PR3 (table 5.1).

This result demonstrated the continued specificity of the probes in assays following PCR amplification with the primer pair ITS3.3/ITS4.2, which had



Figure 5.10. DNA-free samples amplified with primers ITS3.3/ITS4.2 exhibiting contamination from different species. The samples were analysed electrochemically with probe CG PR3 and gave the following signals (1) 0.0nA, (2) 11.1nA, (3) 0.0nA, (4) 0.0nA, (5) 0.0nA and (6) 14.2nA. Lanes 2 and 6 contained amplicon of a size which corresponded to the expected size of the *C. glabrata* product.

never been explicitly tested, and was compelling evidence that a signal would only be produced in the presence of target DNA. This meant that, in assays with any of the probes, DNA-free negative samples which showed contamination could be *a posteriori* regarded as containing DNA from the target species if a positive signal was produced in the electrochemical analysis, thereby rendering the sample a ‘target-containing sample with an indeterminate amount of target DNA’ and such samples could be ignored when assessing the background level of current generated by genuine DNA-free or non-target DNA negative samples. In a similar way, contaminated samples which produce no current can *a posteriori* be considered non-target

Table 5.1. Currents produced by all of the samples used in the assay with CG PR3 which exhibited extensive contamination in the DNA-free samples

Amount of <i>C. glabrata</i> DNA	Sample 1 (nA)	Sample 2 (nA)	Sample 3 (nA)	Sample 4 (nA)	Sample 5 (nA)	Sample 6 (nA)
1.05pg	137.00	124.00	131.00	N/A	N/A	N/A
105fg	111.00	81.90	85.60	N/A	N/A	N/A
10.5fg	64.20	46.70	33.60	10.20	54.80	35.10
DNA-free negative	0.0	11.10	0.0	0.0	0.0	14.20

DNA negative samples if zero current was produced by them in the electrochemical assay. It should be noted that agarose gel analysis of PCR products is not a feature of the assay, but only a checking step for experimental purposes. It does, however, allow deductions such as this to be made. The eventual assay procedure would rely solely on the current production to determine the presence or absence of target DNA and so it was important to establish reliably that any positive signal indicated only the target DNA.

Contamination of samples was a problem in this work for several reasons. As discussed above, continual amplification of *Candida* DNA in the laboratory is likely to have generated much airborne nucleic acid which can easily contaminate PCR samples. The use of generic primers, capable of efficient amplification of target from small DNA amounts, will then amplify even the smallest amounts of contaminating fungal DNA when it is present. However, the above results demonstrate that the specificity of the probes is maintained when target DNA is present in extremely small amounts and the signal produced by the assay is a reliable indicator of the presence or absence of target DNA. It can therefore be reasoned that 'if there is signal then there is target'.

It was considered likely that contamination would continue to materialise during experiments and so assays were altered in light of this to include six replicates of DNA-free samples so that enough non-target or no-amplicon negatives would be produced to give a measure of any background signal

5.5 Detection of template DNA equivalent to one genome.

With the design of more efficient primers, the limitation of contamination and faith in the specificity of the probes, work could now begin in earnest on demonstrating a limit of detection of 1 genome per reaction for each of the target species with the relevant probe.

A 10-fold dilution series of genomic DNA from the four diploid target species (*C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*) was prepared with

concentrations ranging from $3.7\text{pg } \mu\text{l}^{-1}$ – $37\text{fg } \mu\text{l}^{-1}$ (equivalent to ~ 100 genomes μl^{-1} to ~ 1 genome μl^{-1}). Another 10-fold dilution series of genomic DNA with concentration ranging between $1.05\text{pg } \mu\text{l}^{-1}$ and $10.5\text{pg } \mu\text{l}^{-1}$ (equivalent to ~ 80 genomes μl^{-1} to ~ 0.8 genomes μl^{-1}) was prepared for the haploid species *C. glabrata*. The intention was originally to make a *C. glabrata* DNA dilution series from $1.5\text{pg } \mu\text{l}^{-1}$ to $15\text{fg } \mu\text{l}^{-1}$ but a miscalculation was made when diluting the stock solution of DNA which led to the preparation of the above dilutions. PCR reactions were prepared and $1\mu\text{l}$ of the template DNA solutions were used to amplify target DNA for electrochemical analysis with the relevant species' probe.

5.5.1 Detection of *C. albicans* DNA.

Fig 5.11 shows the mean currents produced by the assay with the *C. albicans* DNA dilutions series and probe CA PR3. The mean current ranged from $233.7 \pm 6.0\text{nA}$ with 3.7pg of DNA to $80.5 \pm 8.7\text{nA}$ with 37fg of DNA and all six DNA-free samples gave zero currents. This was an excellent result and demonstrated that detection of DNA equivalent to a single genome of *C. albicans* present in a $50\mu\text{l}$ reaction could be successfully detected.

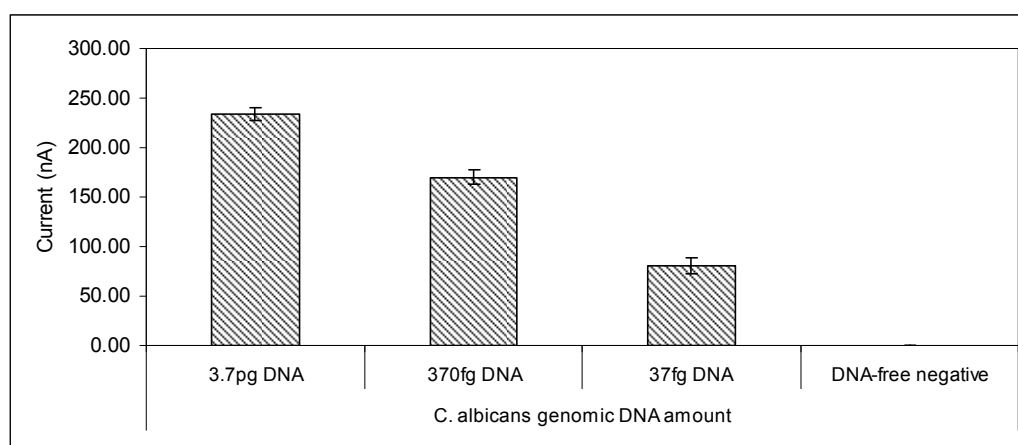


Figure 5.11. Mean currents obtained in assays with CA PR3 and target DNA derived from low amounts of genomic DNA. The error bars represent SD ($n=3$ for assays using 3.7pg and 370fg DNA and $n=6$ for assays with 37fg DNA and DNA-free samples).

5.5.2 Detection of *C. parapsilosis* DNA.

Figure 5.12 shows the mean currents produced by assays with *C. parapsilosis* DNA dilutions and the probe CP PR. Mean currents ranged from 261.0 ± 64.5 nA for assays using 3.7pg DNA to 54.6 ± 27.1 nA for assays using 37fg DNA. The relatively large value obtained for the standard deviation of the currents obtained at 37fg was as a result of two high outlying currents of 65.0nA and 87.3nA. This high variation was probably due to the PCR amplification step being unable to generate reproducible amounts of target DNA when low amounts of genomic DNA were used as target for the reaction. Nonetheless, the results demonstrated that DNA equivalent to a single genome of *C. parapsilosis* could be detected in a 50µl reaction.

5.5.3 Detection of *C. glabrata* DNA.

Figure 5.13 shows the mean currents obtained in the assays with low amounts of *C. glabrata* genomic DNA. The mean currents ranged between 130.7 ± 6.5 nA at 1.05pg DNA to 40.8 ± 19.0 nA at 10.5fg DNA. There was again a relatively high standard deviation observed when a very low amount of DNA (10.5fg) was used as template for the reaction. This was due to a low current of 10.2nA which corresponded to a very weak PCR product and two other relatively low currents of 33.6nA and 35.1nA which corresponded to PCR products which contained a contaminant amplicon; the low signals may have been due to a lower amount of target being produced because of competition for PCR reagents by two different sources of DNA. The results did however demonstrate that the assay could detect *C. glabrata* DNA down to 10.5fg (~0.8 genomes) in a 50µl reaction.

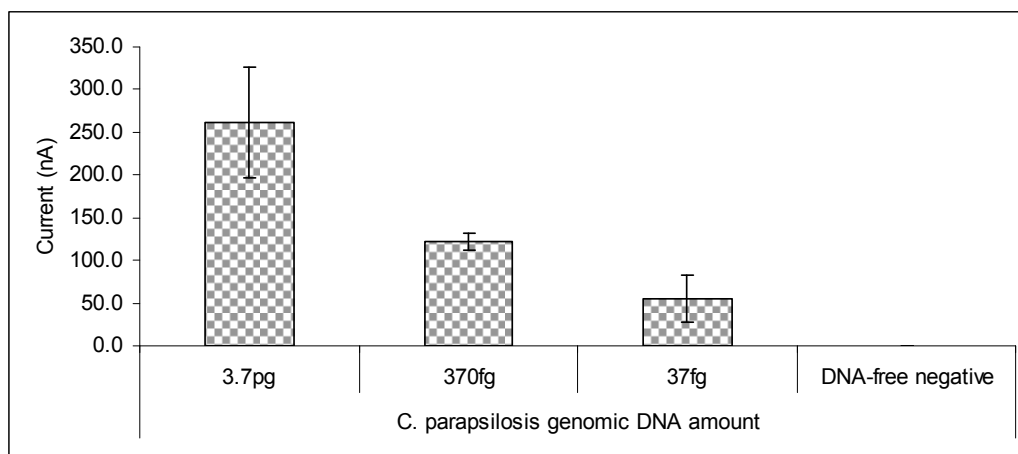


Figure 5.12. Mean currents obtained in assays with CP PR and target DNA derived from low amounts of genomic DNA. The error bars represent SD (n=3 for assays using 3.7pg and 370fg DNA, n=4 for assays with 37fg DNA and n=5 for assays with the DNA-free samples).

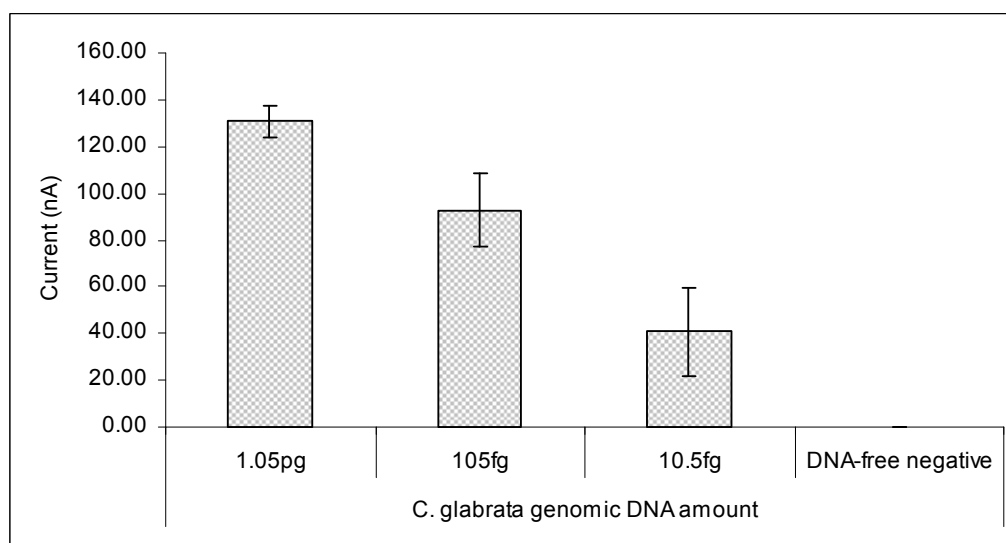


Figure 5.13. Mean currents obtained in assays with CG PR3 and target DNA derived from low amounts of genomic DNA. The error bars represent SD (n=3 for assays using 1.05pg and 105fg DNA, n=6 for assays with 10.5fg DNA and n=4 for assays with the DNA-free samples).

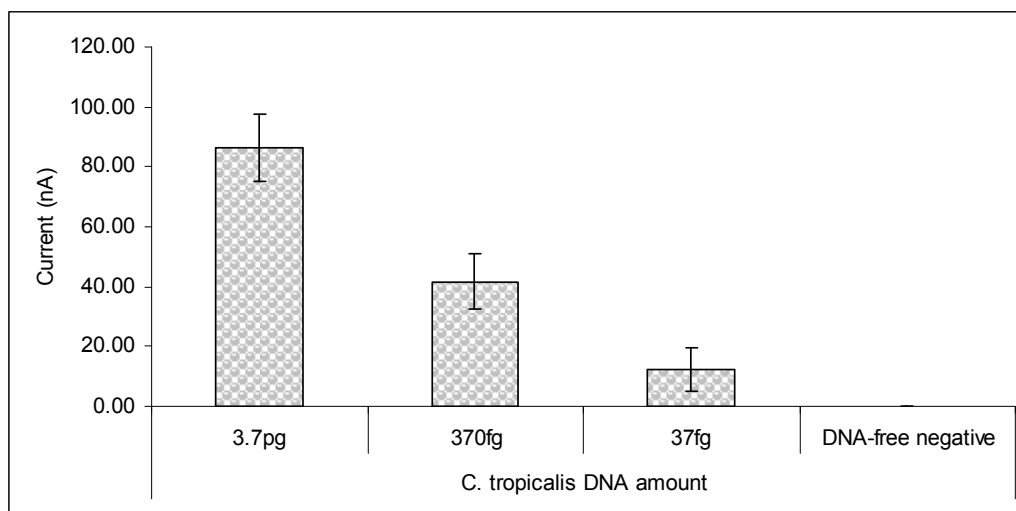


Figure 5.14. Mean currents obtained in assays with CT PR and target DNA derived from low amounts of genomic DNA. The error bars represent SD (n=3 for assays using 3.7pg and 370fg DNA, n=6 for assays with 37fg DNA and with the DNA-free samples).

5.5.4 Detection of *C. tropicalis* DNA.

Figure 5.14 shows the results of assays with low amounts of *C. tropicalis* DNA. The mean currents obtained ranged between 86.3 ± 11.1 nA at 3.7pg DNA and 12.2 ± 7.3 nA at 37fg DNA. Again, the standard deviation obtained at the lowest DNA amount was relatively large and there was nothing anomalous about the PCR products. This was further evidence that the PCR amplification was much less reproducible when extremely small amounts of DNA were added to the reaction. It had also been found that CT PR can potentially form an extensive hairpin structure involving its 5' terminus (fig 5.15) which may have proved detrimental to the current produced by the assay. Despite this, the detection of low amounts of *C. tropicalis* DNA was shown to be achieved by the probe CT PR.

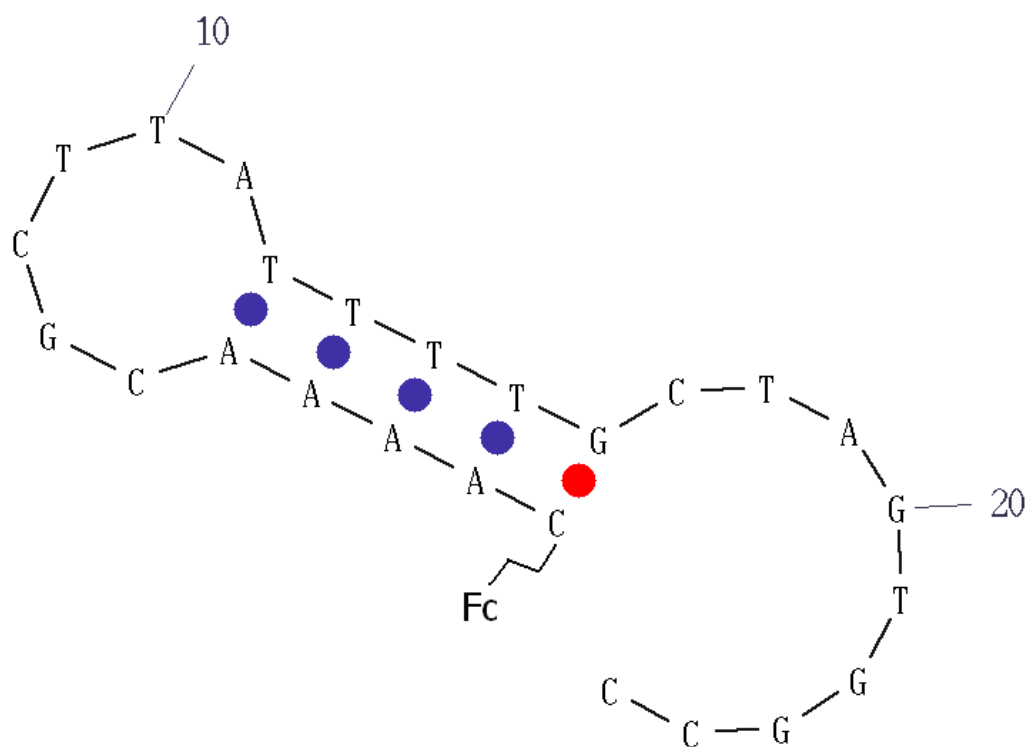


Figure 5.15. Potential hairpin structure of probe CT PR. Fc represents the ferrocene label attached at the 5' end of the oligonucleotide.

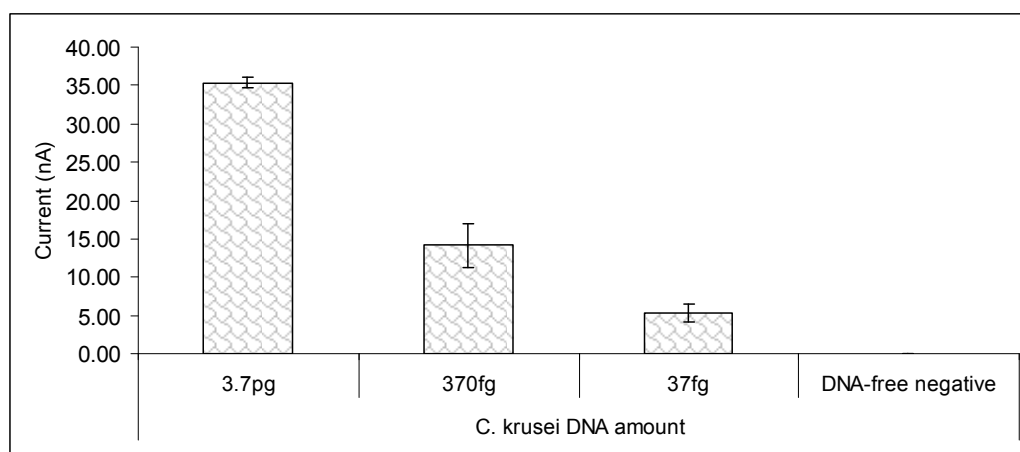


Figure 5.16. Mean currents obtained in assays with CK PR and target DNA derived from low amounts of genomic DNA. The error bars represent SD (n=3 for assays using 3.7pg and 370fg DNA, n=5 for assays with 37fg DNA and n=6 for assays with the DNA-free samples).

5.5.5 Detetction of *C. krusei* DNA.

Figure 5.16 shows the results of assays using the dilution series of *C. krusei* DNA. The mean currents obtained were very low compared with the signals of the other probes, ranging between 35.4 ± 0.7 nA obtained with 3.7pg DNA to 5.3 ± 1.1 nA obtained with 37fg DNA. The standard deviations were also considerably smaller with these results and it was not clear why. Nonetheless, despite the very small currents generated with 37fg DNA the fact that the DNA-free background was zero meant that it could be regarded as positive detection of *C. krusei* DNA down to a single genome equivalent in a 50µl reaction.

5.5.6. Limit of detetction of PanF PR probe with *C. albicans* DNA.

The PanF PR probe was tested using a *C. albicans* DNA dilution. The mean currents produced (fig 5.17) were 188.7 ± 58.0 nA with 3.7pg DNA, 121.7 ± 11.1 nA with 370fg DNA and 40.4 ± 12.8 nA with 37fg DNA; the background current was 0 nA. This was another excellent result with comparatively high mean currents generated at all DNA amounts tested. As only *C. albicans* DNA was tested, the result could not however be assumed to hold for all other fungal species as there may be specific differences that would result in a lower signal being produced and this would have to be tested

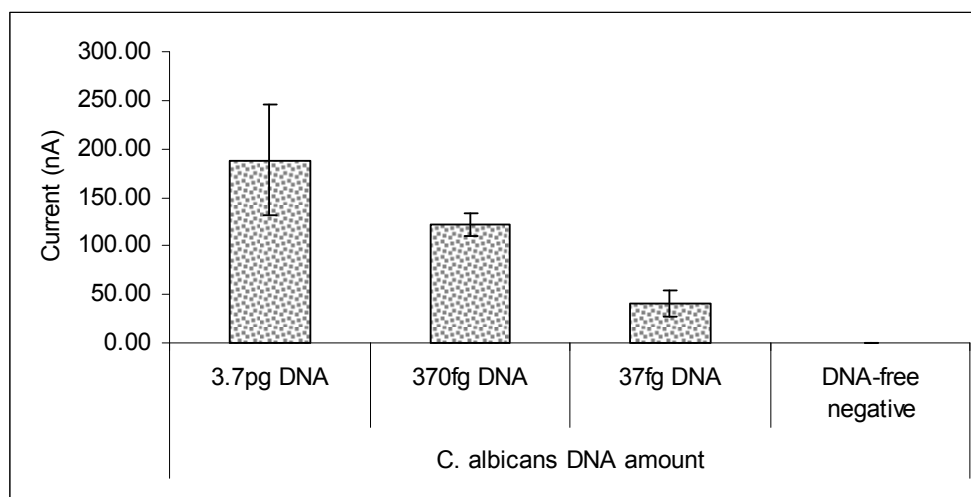


Figure 5.17. Mean currents produced with probe PanF PR in assays with *C. albicans* DNA. Error bars represent SD (n=3 for 3.7pg and 370fg samples and n=6 for 37fg and DNA-free samples).

experimentally. Given the high mean currents produced by PanF PR in assays with 15 fungal species (section 4.4) it was likely that the probe would be able to detect low amounts DNA from most fungal species.

5.6. Conclusions.

Detection of DNA equivalent to one genome in a 50 μ l reaction was shown to be possible for all of the probes. Establishing the limit of detection was made difficult by inefficient primers and the presence of contaminating DNA. However, following the redesign of primers and the preparation of reagents in a cleaner environment, the assay was shown to be robust enough to detect target DNA when it was present in the reaction at low amounts.

The signals generated by each probe varied and the probes CA PR3, CP PR and CG PR3 gave the highest mean currents with a single genome of 80.5 ± 8.7 nA, 54.6 ± 17.1 nA and 40.8 ± 19.0 nA respectively. The *C. krusei* specific probe CK PR gave the poorest results with 1 genome giving a mean current of only 5.3 ± 1.1 nA. This was a particularly weak signal and was only considered successful detection by virtue of the zero background level produced by the new PCR conditions. This further highlighted the need for redesign of the *C. krusei* probe sequence. It was also noted that the CT PR probe gave a relatively low signal of only 12.2 ± 7.3 nA. As noted above, the probe is potentially capable of forming secondary structures involving its 5' end which may have affected its digestion and decreased the current produced. It was likely that this probe could also be redesigned in an effort to obtain improved results more in line with the other probes.

The results would have been improved by replication with a greater number of samples to further validate the assay's ability to detect small amounts of target DNA. This was not attempted here as all that was required was a demonstration of the assay's ability to detect a single genome in a reaction to allow the project to progress to detection of a low amount of DNA from a spiked 1ml blood sample.

Chapter 6 - Detection of fungal DNA from blood.

6.1 Introduction

The previous chapters have described the optimisation of the fungal probe detection suite using extracted DNA added directly to the PCR reaction. In order to validate the assay further, detection of fungal DNA must be achieved from blood samples.

Blood is a difficult medium to work with as it contains a variety of components – erythrocytes, leukocytes, platelets and a host of salts, sugars, proteins and other macromolecules. Some of these compounds affect PCR by inhibiting *Taq* DNA polymerase while the leukocyte population of cells represents a large source of human DNA which may inhibit PCR by competing non-specifically for primers. Because the fungal load of a candidaemic individual can be as low as 5 c.f.u ml⁻¹, detection of fungal DNA in blood is difficult (Loeffler *et al.*, 2000).

For successful amplification of DNA from blood an effective extraction protocol is necessary whereby the human cells are lysed and all of the potentially inhibitory macromolecules present in the blood lysate are removed from the sample. Additionally, the fungal cells must themselves be lysed to release their genomic DNA. These tasks require two separate treatments, further complicating the business of DNA extraction for detection of fungi. Furthermore, the procedure must be capable of recovering minute amounts of fungal DNA in a background of proportionally vast amounts of human DNA.

Blood can be processed into three different fractions: whole blood is the 'crude' sample which contains all of the cellular and macromolecular constituents and is the 'dirtiest' sample. Plasma is the supernatant obtained following centrifugation of an uncoagulated whole blood sample to pellet the cells – it retains the dissolved salts, macromolecules and clotting factors. Serum is similar to plasma but lacks the clotting factors as it is prepared by allowing the blood to coagulate before centrifugation. The clot is then pelleted

and the serum supernatant can be extracted. For this reason serum is the 'cleanest' sample but is still rich in proteins, salts and sugars.

The choice of which fraction to use for a DNA based diagnostic assay is influenced by the balance of recovery of nucleic acid vs. contamination by molecules inhibitory to PCR amplification of target DNA (note that DNA from leukocytes could be included in this category). Fungal cells will obviously be found in the cell fraction of a blood sample and so use of plasma and serum fractions for detection of DNA risks eliminating a primary source of fungal DNA from a sample. Exogenous DNA from damaged fungal cells may be present in plasma and serum samples, but the lifespan of such DNA molecules is uncertain since DNase activity has been reported in both serum and plasma fractions of human blood samples (Cherepanova *et al.*, 2007). Molecular assays have been shown to successfully detect fungal DNA from clinical samples of blood serum (McMullan *et al.*, 2008) and studies comparing the detection of fungal DNA in whole blood, plasma and serum have shown that detection may in fact be more reliable when plasma or serum samples are used (Bougnoux *et al.*, 1999; Metwally *et al.*, 2008; Lau *et al.*, 2009) although the number of samples tested was relatively small.

The researchers at Atlas Genetics developed a prototype DNA extraction protocol for use on bacterial pathogens present in clinical samples based on the Sigma Genelute column extraction kit. This was a relatively rapid and low-tech method for extraction of nucleic acid from samples and it was decided to try and adapt this method for extraction of fungal DNA from spiked blood samples.

6.2 Extraction of nucleic acid from fungal DNA-spiked whole blood and plasma samples

Blood was acquired from a healthy volunteer and deposited into vacutainers and processed to yield plasma, serum and whole blood fractions as described in Chapter 2. For the initial experiment to test the extraction procedure three 1ml samples of whole blood and plasma were used. The reason for excluding serum samples from this first test was solely due to the

difficulty of processing large numbers of samples due to the multiple centrifugation steps and handling of a biohazardous material.

The 1ml samples were prepared in different ways in order to test different factors of the extraction procedure. Samples of whole blood and plasma were kept free of *C. albicans* DNA, another sample of each had 37pg of *C. albicans* DNA (~1000 genomes) added before the extraction procedure, and the third aliquot of each sample was kept free of *C. albicans* DNA until after the extraction procedure when 37pg of *C. albicans* DNA was added to the eluted material. These conditions would allow the effect of the background human DNA to be measured in isolation and would also show if there was any difference between fungal DNA recovered from a blood sample compared with the same amount added to the eluate from an unspiked blood sample.

Once the extractions were complete three 30µl aliquots of each of the 100µl eluates was added to 20µl of a PCR mix to give three replicates for each set of DNA extraction conditions. Positive control samples with 37pg of extracted *C. albicans* DNA added to the PCR were set up to allow comparison of the PCR products generated from DNA extracted from blood.

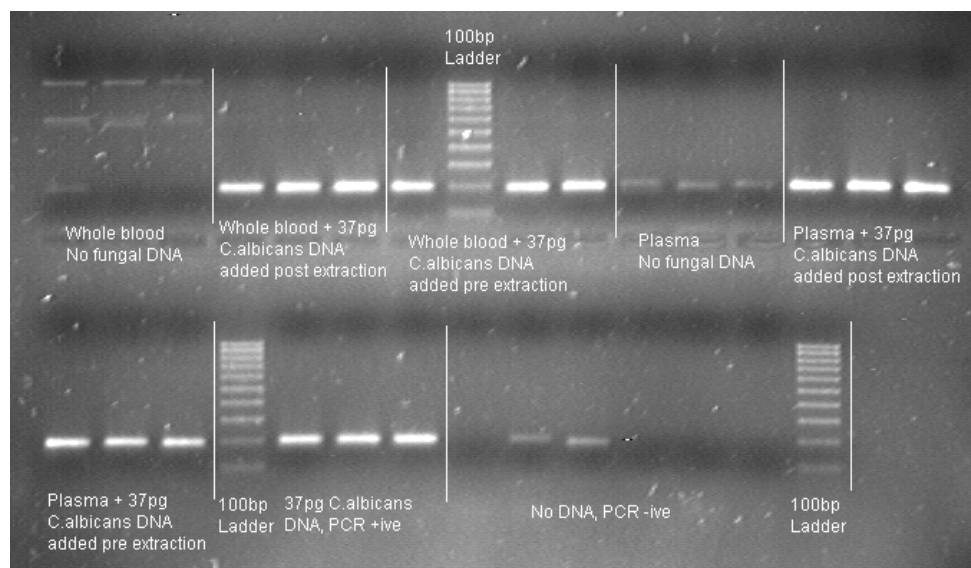


Figure 6.1. PCR products from amplifications with extracted products from spiked and unspiked whole blood and plasma samples.

The DNA was amplified and the products were run on an agarose gel to check the products (fig 6.1).

All of the samples that were spiked with *C. albicans* DNA produced amplicon of similar intensity to the PCR positive control, indicating that the extraction method was efficient at recovering a large amount of *C. albicans* DNA from blood. The unspiked whole blood samples all had evidence of non-fungal PCR products of ~600bp and ~1000bp which were likely to have originated from the human DNA fraction while one sample contained a third amplicon which was very likely to be fungal in origin indicating that contamination may have occurred. The spiked whole blood samples contained single amplicons corresponding to the expected size of the *C. albicans* product, suggesting that the *C. albicans* DNA had out-competed the human genomic DNA for the ITS3.3/ITS4.2 primers. There were signs of fungal contamination in all of the reactions that were set up using material that had been extracted from the unspiked plasma samples, as well as in two of the DNA-free negative samples. Given the relative difficulty involved in carrying out the DNA extractions from blood, rather than repeat the extraction

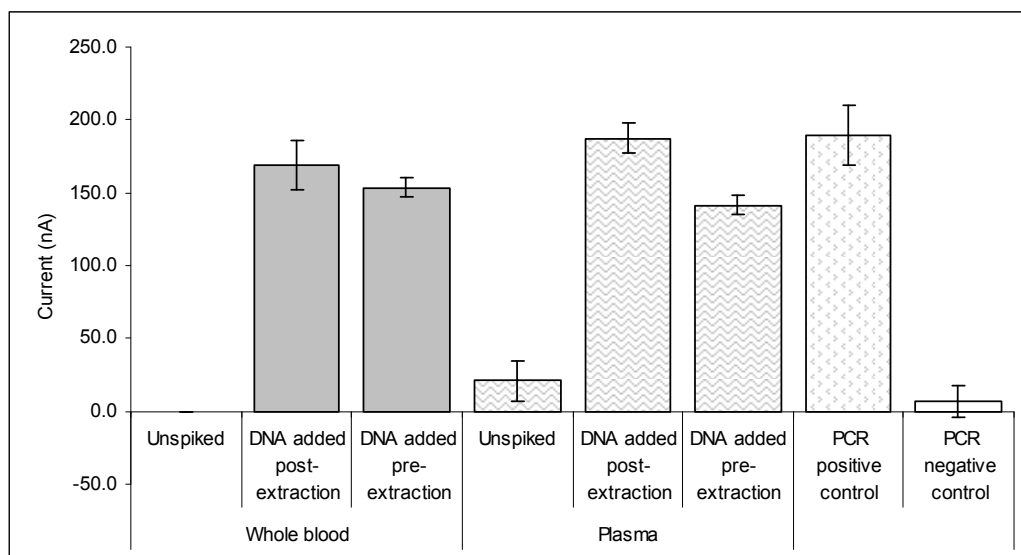


Figure 6.2. Mean currents produced in assays using DNA extracted from spiked and unspiked whole blood and plasma samples. Error bars represent SD (n=3 for all results except for the PCR negative control, where n=6).

to obtain contamination-free results the PCR products were analysed electrochemically using probe CA PR3 in spite of the evident contamination. The results are shown in fig 6.2. The mean current produced by the positive control samples was $189.9 \pm 20.7\text{nA}$. Two of the negative control samples produced positive signals while the other four gave zero currents, yielding a mean of $7.0 \pm 11.2\text{nA}$. All of the unspiked plasma samples produced current which resulted in a mean of $21.1 \pm 13.8\text{nA}$ indicating that the samples had been contaminated by a small amount of *C. albicans* DNA. The whole blood and plasma samples which had been spiked prior to DNA extraction gave good levels of signal, $153.4 \pm 6.6\text{nA}$ and $141.4 \pm 6.6\text{nA}$ respectively, indicating positive detection of *C. albicans* DNA in these samples. The blood and plasma samples which had *C. albicans* DNA added to the extraction eluate produced slightly higher mean currents compared to the pre-extraction spiked products: $168.9 \pm 17.1\text{nA}$ for whole blood and $187.5 \pm 10.3\text{nA}$ for plasma. The difference was very small, however, and was probably due to the loss of fungal DNA due to the inefficient nature of the column-based DNA extraction method used. There was certainly no evidence of inhibitory substances being copurified with the DNA in either the whole blood or plasma samples.

These results indicated that there was little difference between mean currents produced by whole blood or plasma that had been spiked with *C. albicans* DNA. The experiment was not repeated with serum samples as it was thought unlikely that the removal of clotting factors from a plasma sample would have much effect on the current produced by the assay. It was also decided that, given the ease of preparation of whole blood samples, all further work to detect low amounts of *C. albicans* DNA in blood would use whole blood samples.

6.3. Optimisation of assay conditions for detection of ~1 genome *C. albicans* DNA in 1ml blood samples.

6.3.1 Establishing limit of detection of *C. albicans* extracted DNA in blood.

Following the success of detecting a relatively large amount of *C. albicans* DNA spiked into 1ml blood samples the next step was to determine the limit of detection of DNA extracted from blood and, if possible, optimise the extraction procedure to achieve a limit of detection of 1 genome ml⁻¹ of blood. The first attempt used 1ml blood samples spiked with 3.7pg *C. albicans* DNA (~100 genomes), 370fg *C. albicans* DNA (~10 genomes) and 37fg *C. albicans* DNA (~1 genome). The samples were eluted in 100µl buffer as before and 30µl of each eluate was used as template in PCR amplifications. The products obtained can be seen in figure 6.3 and show the presence of additional bands in samples using DNA extracted from blood. The bands are similar to those observed in the unspiked whole blood samples in figure 6.1 and are almost certainly linked to the presence of extracted human DNA in these samples. One possible explanation is that they are amplicons produced by priming of human DNA by the ITS3.3/4.2 primer pair. It can be inferred that the primer binding sites have only partial similarity as the bands are relatively weak and are only observed when competition for the primers by *C. albicans* DNA is low i.e. when the amount of *C. albicans* DNA present in the reaction is less than or equal to 3.7pg. The positive control and the samples containing ~100 and ~10 genomes produced an amplicon that corresponded to the expected size of *C. albicans* but the samples containing ~1 genome showed the presence of only the human-related amplicon. It was possible that sub-visual amplicons were present in these samples and would be detected when the samples were analysed electrochemically. Of the six DNA-free negative controls one showed a contaminant amplicon which was slightly larger than the expected size of *C. albicans* and so, as a non-target amplicon, was likely to produce a zero current in the assay.

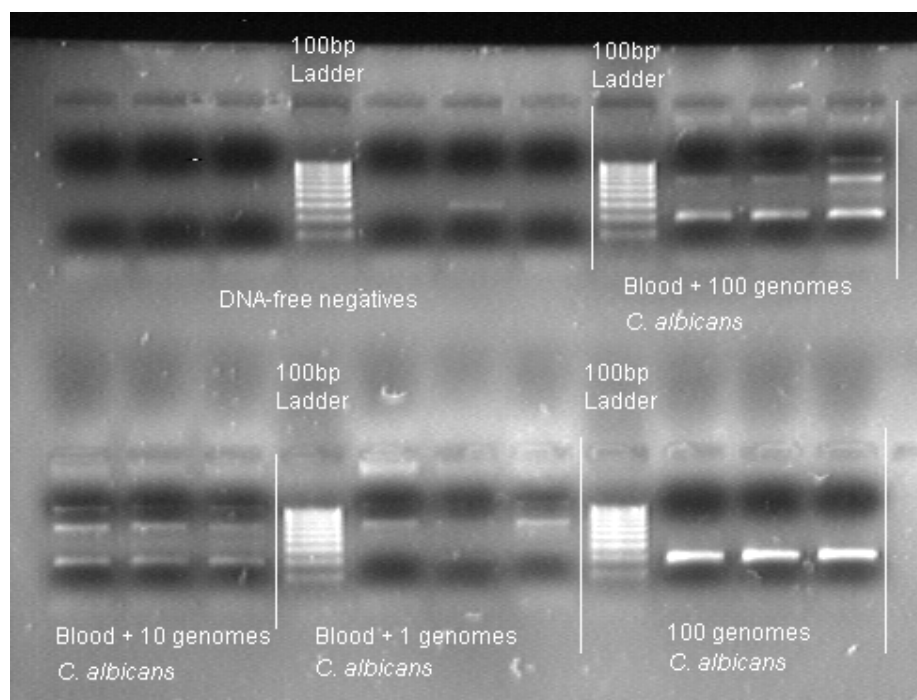


Figure 6.3. PCR products obtained with *C. albicans* DNA extracted from 1ml blood samples.

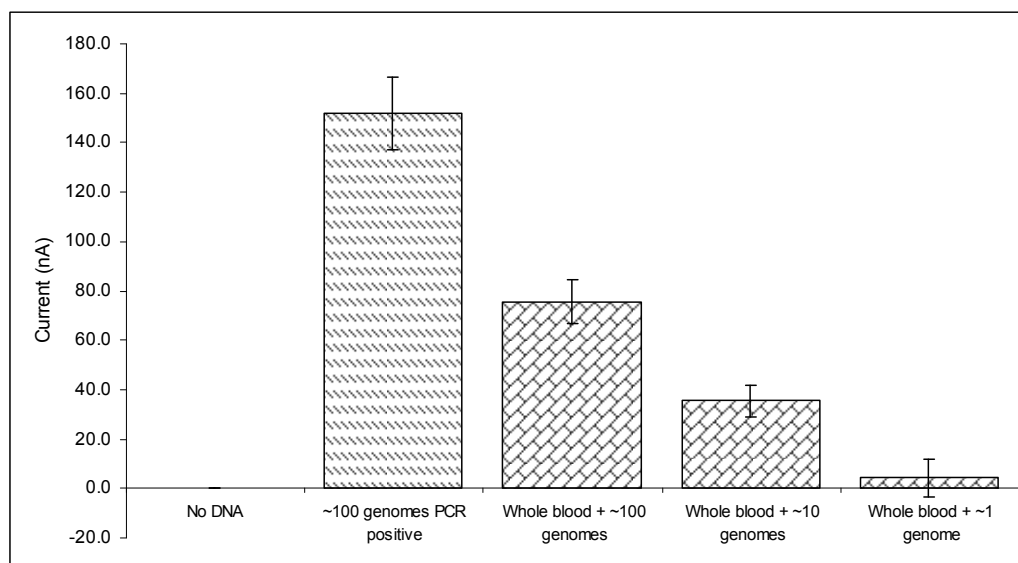


Figure 6.4. Mean currents obtained with low amounts of *C. albicans* extracted DNA spiked into 1ml blood samples. Error bars represent SD ($n=6$ for DNA-free samples and $n=3$ for all other samples).

The PCR products were analysed electrochemically using CA PR3 and the mean currents are shown in fig 6.4. The positive control of ~100 genomes of extracted DNA in the PCR reaction gave a mean current of $152.0 \pm 14.8\text{nA}$ compared to means of $75.6 \pm 8.9\text{nA}$, $35.6 \pm 6.4\text{nA}$ and $4.4 \pm 7.6\text{nA}$ for ~100, ~10 and ~1 genomes spiked into whole blood respectively. All six of the DNA free samples gave zero current.

The reason for the difference in signal between the positive control sample containing ~100 genomes and the sample containing extracted material from a blood sample spiked with ~100 genomes is likely to be due to several factors. Firstly, no extraction process is 100% efficient and this would result in a loss of DNA; secondly, of the 100 μl of eluted DNA only 30 μl is used as template in each PCR replicate so it is likely that only ~300fg is present in each reaction; and finally, there is a large amount of human DNA that co-elutes with the *C. albicans* DNA and, as discussed above, this would appear to compete for PCR reagents. According to this reasoning, these factors would reduce the amount of target DNA produced by PCR and hence reduce the current produced in the assay.

Nonetheless, all of the samples containing template extracted from spiked blood samples containing ~100 and ~10 genomes gave consistent currents in the assay. The assays which used template from blood spiked with ~1 genome produced current in only one out of the three replicates, indicating that the method was not capable of reliable detection of this level of fungal DNA. When the amount of fungal DNA in a 1ml blood sample is as low as ~1 genome the effects described above would have a much greater effect on the amount of DNA available for PCR. The practice of eluting DNA in 100 μl and then splitting this into three fractions to use as template for PCR was particularly unsatisfactory in this respect.

6.3.2 Adapting extraction protocol to improve detection of ~1 genome.

The most obvious stage in the extraction procedure at which a large amount of DNA was lost was when the 100 μl eluate was split into three fractions to produce triplicate samples of template for PCR. An obvious way

to improve the extraction procedure was therefore to use a smaller volume for elution of DNA and use all of this extracted material as template for PCR. This solution was not without potential problems, however, as decreasing the volume of eluent is known to result in a reduced yield of DNA. Nevertheless, this adaptation to the extraction procedure was attempted and DNA from three separate 1ml blood samples spiked with ~1 genome of *C. albicans* DNA was eluted in 30µl buffer to provide material for three replicate assays.

Figure 6.5(A) shows the PCR products where it can be seen that only one of the reactions using DNA extracted from the spiked blood samples gave a visible PCR product. This sample contained two products one of which was of the expected size for *C. albicans* (~200bp) while the other was likely to be a fungal contaminant (~320bp). The samples were analysed electrochemically and the mean currents can be seen in fig 6.5(B). The PCR positive containing ~1 genome added directly to the PCR reaction gave a mean signal of 85.3 ± 13.6 nA compared to a mean of 15.0 ± 17.2 nA produced by the samples extracted from blood. The large standard deviation was caused by the widely varying production of current in the three samples: 0nA, 33.8nA and 11.1nA. The adapted extraction protocol had apparently improved detection since 2 out of 3 samples gave a signal compared with 1 out of 3 using the original protocol, but it was evidently still not capable of yielding sufficient target material to allow reliable detection of ~1 *C. albicans* genome in 1ml blood and further optimisation was required.

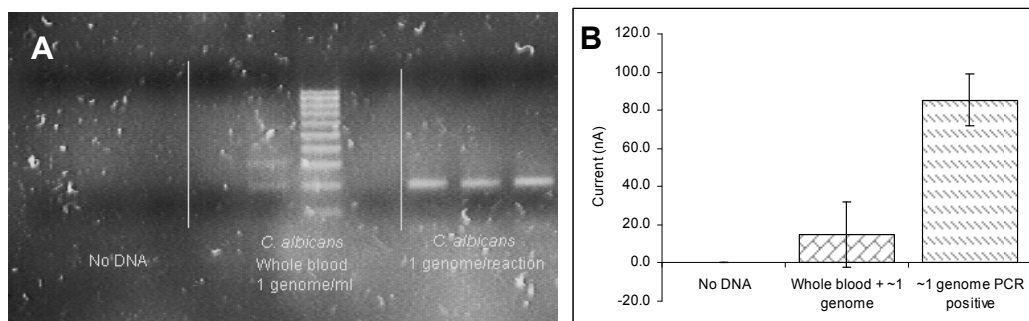


Figure 6.5. (A) PCR products obtained using 30µl of eluted DNA from blood samples spiked with ~1 genome *C. albicans* DNA. (B) Mean currents obtained in assays using the PCR products. Error bars represent SD (n=3).

Upon consulting with Sigma-Aldrich about the GenElute kit it was suggested that heating the elution buffer to 60°C before applying it to the extraction column and applying the eluate to the spin column a second time may increase the yield of DNA. The amount of elution buffer used was also increased to 34.25µl which was the maximum amount that could be added to the PCR mix. Total DNA was extracted from 1ml blood samples spiked with ~1 genome of *C. albicans* DNA by a protocol incorporating the above changes and was used as template in PCR.

The PCR products were considerably better using this extracted material as template. Figure 6.6 demonstrates that all of the samples containing material extracted from spiked blood samples gave a visible amplicon of the expected size for *C. albicans*. Furthermore there was a clearly visible smear of high molecular weight DNA, likely to be total genomic DNA; such a smear had also been observed in figure 6.3 but was much weaker. This was further evidence that the DNA extraction technique had been much improved by the changes to DNA elution. There was evidence for a second, smaller amplicon in the second and third samples, as well as the second PCR positive sample suggesting that these samples may have been contaminated. The DNA-free samples were all clear, however, indicating that contamination was not a systemic problem in the experiment.

The samples were analysed electrochemically and the mean currents obtained are displayed in figure 6.7. The PCR positive gave a mean current of $58.0 \pm 18.2\text{nA}$ compared to $26.3 \pm 14.3\text{nA}$ obtained with DNA extracted from spiked blood samples. It was worth noting that the lowest signal produced was 13.6nA and this was obtained with the extracted sample that contained the strongest contaminant amplicon. It was possible that competition for PCR components had led to a reduction in the signal.

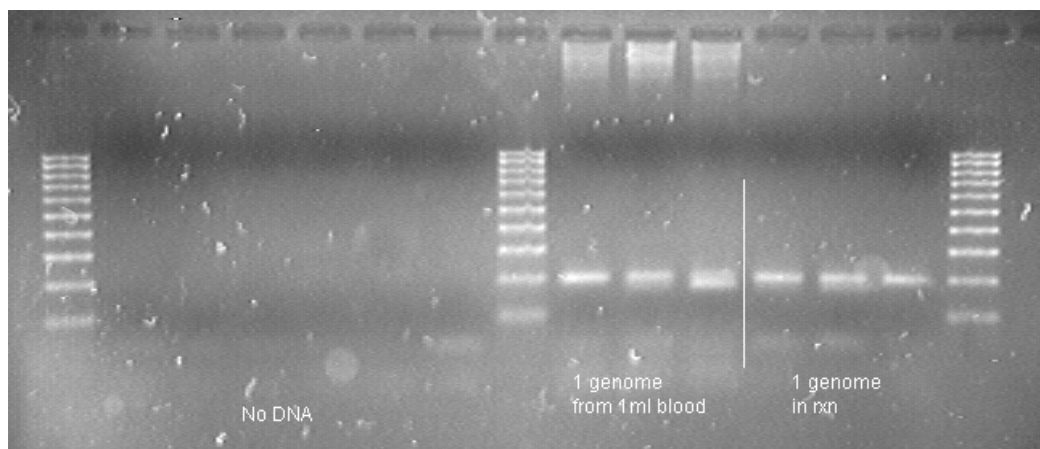


Figure 6.6. PCR products obtained using 34.25 μ l of eluted total DNA from blood samples spiked with \sim 1 genome *C. albicans* DNA. Eluate was passed through the column twice to enhance yield.

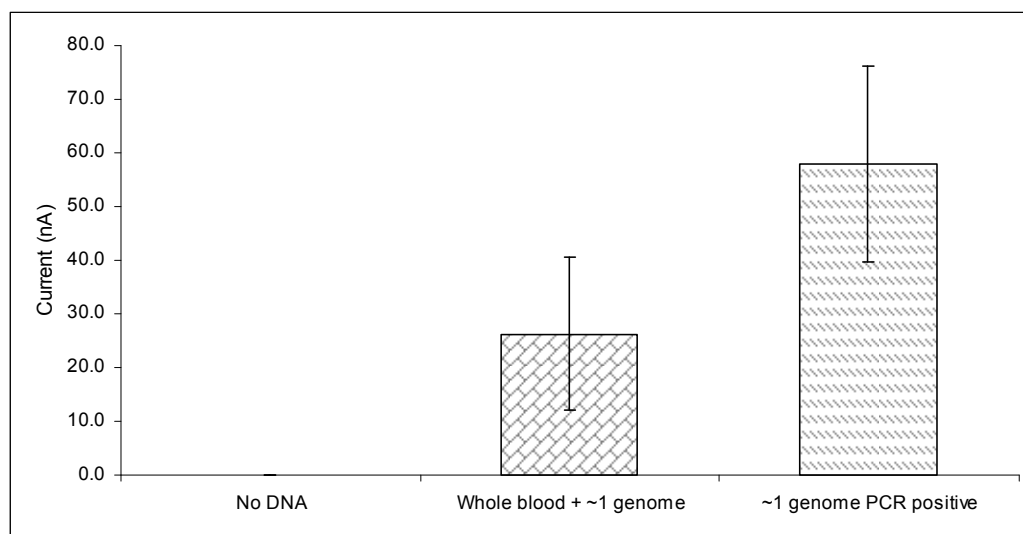


Figure 6.7. Mean currents obtained in assays using the PCR products generated by the enhanced extraction procedure. Error bars represent SD (n=3).

Nonetheless, the difference observed between the means of the PCR positive and the extracted samples demonstrated that the improved DNA extraction procedure was still far from perfect. On the other hand, the procedure had resulted in all three extracted samples producing a current, indicating that the improved procedure had considerably increased the reliability of detection of ~1 genome of *C. albicans* DNA in 1ml of blood.

6.4. Limit of detection of whole cells of *C. albicans* in blood.

Following the optimisation of DNA extraction for detection of a single genome of *C. albicans* DNA spiked into 1ml blood samples the next obstacle was to try and extract and detect DNA from a single *C. albicans* cell in 1ml blood samples. Extracting DNA from intact cells requires further treatment of samples as the yeast cells need to be lysed to release DNA. The existing extraction protocol includes a chemical lysis step using detergent and guanidine thiocyanate to disrupt lipids and membrane proteins of human cells but it was not known if this would be sufficient to lyse the complex cell walls of yeast. Options for lysis of yeast cells include physical, chemical and enzymatic methods. A chemical lysis using NaOH was initially attempted but was unsuitable as it caused coagulation of blood and loss of total DNA (data not shown). Of the remaining options the enzymatic approach was favoured.

6.4.1. Enzymatic lysis of fungal cells prior to extraction

An enzymatic lysis step using 5U of lyticase was incorporated into the DNA extraction procedure. DNA was extracted from 1ml blood samples spiked with either 10 *C. albicans* cells (n=1) or 1 *C. albicans* cell (n=3). The 10-cell sample was eluted in 105µl elution buffer which was split into three fractions of 34.25µl to be used as template for PCR while all three of the single cell samples were eluted into 34.25µl and used as template for PCR. The amplification of the extracted products was not successful (fig 6.8) with only one amplicon of the appropriate size being produced by the samples

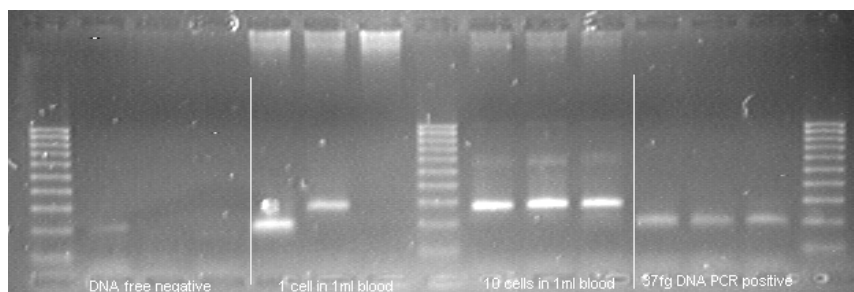


Figure 6.8. PCR products obtained using material extracted from 10 cell and 1 cell-spiked blood samples.

containing template from the 1 cell-spiked samples and none at all from the 10 cell extracts. The latter samples also showed contamination by an ~280bp amplicon which was evidently present in the initial eluate and, following splitting of the 105µl eluate into three fractions, it was present in all three PCR amplifications. A similar sized contaminant was also observed in one of the samples amplified from DNA extracted from a 1 cell-spiked sample which showed that the contaminant, while not systemic, was occurring with relatively high frequency.

The contaminant product was sequenced to establish its identity and, although the electropherogram was messy, the sequence had a high similarity to *Saccharomyces cerevisiae*. The presence of DNA from this yeast in the PCR amplifications was difficult to account for as it was not routinely used in the Atlas Genetics lab where the DNA extraction and PCR was performed. The most likely source of contamination was from the laboratory at the University of Bath where the stock primer solutions and the initial preparation of yeast cell suspensions was made, although an environmental source at the Atlas Genetics laboratory could not be ruled out.

The only *C. albicans*-sized amplicon produced by template from the 1 cell-spiked material appeared to be stronger than expected - it was of a greater intensity than amplicons produced by conventionally extracted DNA equivalent to 1 genome. A weakly amplified *C. albicans*-sized product was also observed in one of the DNA-free samples.

Upon electrochemical analysis of the products, currents were produced by all samples with a *C. albicans*-sized product: 18.9nA for the contaminated DNA-free sample, 52.5nA for the 1 cell-spiked sample and values of 66.1nA, 39.0nA and 55.3nA for the PCR positive samples. The similarity of the value produced by the 1 cell-spiked sample to the control samples was further indication that it was likely to be an anomalous result as all previous work with DNA extracted from 1 genome-spiked samples had resulted in signals lower than the PCR positive control.

The initial attempt to extract DNA from small numbers of yeast cells spiked in blood had not been successful. It seemed logical, following the failure of the above experiment, to focus on achieving lysis of 10 *C. albicans* cells initially, before trying to lyse and extract DNA from single cells spiked into blood. Furthermore, it was apparent that improved results could be obtained by altering the extraction procedure so that elution of DNA in a preheated volume of 34.25µl elution buffer was performed as standard, regardless of how much *C. albicans* DNA had been spiked into the sample.

6.4.2 Detecting DNA extracted from a suspension of 10 *C. albicans* cells

Following from these proposed improvements, a repeat of the above yeast-cell lysis and DNA extraction procedure was attempted in the absence of blood using a 10µl suspension of 10 *C. albicans* cells only. Three separate extractions were performed and DNA eluted in the smaller volume recommended above to obtain template DNA for three PCR amplifications.

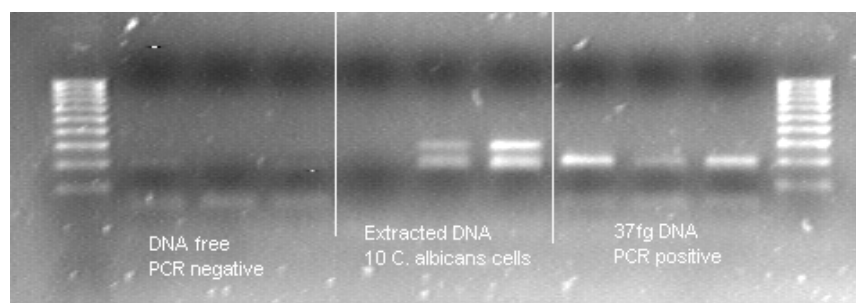


Figure 6.9. PCR products obtained using extracted DNA from three separate 10 cell-spiked blood samples. Note the very weak amplicon present in the first of the DNA-free samples.

The extracted samples gave improved PCR products (fig 6.9), with two out of three samples using DNA extracted from 10 cells producing amplicon. Unfortunately these products also contained contaminant amplicons of ~280bp similar to those observed before. It was noteworthy that the contaminant should only appear in the samples which also produced target amplicon as it suggested that total DNA may have been somehow lost from the first extracted sample.

The products were analysed electrochemically and showed that the first DNA-free sample had been contaminated by DNA from *C. albicans* (21.9nA signal) and that the two extracted samples that had produced amplicon did indeed contain *C. albicans* DNA, giving signals of 17.8nA and 21.8nA. These results compared with a mean current produced by the PCR positive control of 50.7 ± 11.4 nA, obtained using 1 genome as template indicating that a relatively large loss of signal may have been occurring in the extraction procedure of 10 cell-spiked samples. The presence of contaminant DNA may also have been a factor, however, as this would have competed for PCR reagents and inhibited amplification of the target DNA. However the results did demonstrate that detection was possible, if not reliable.

An important point to note is that the electrochemical signal produced by PCR products from extracted genomic DNA are not directly comparable to signals produced by DNA extracted from cells. A better control would have been 10 *C. albicans* cells added directly to the PCR. Numerous attempts were made to amplify DNA from 10 cells added directly to the PCR but no amplification was ever observed (data not shown). An alternative control would have been DNA that had been extracted from a suspension 10 *C. albicans* cells in the absence of blood but this was not considered until after the experiments.

The above procedure was applied to spiked blood samples to ascertain its reliability. Six 1ml blood samples were spiked with a 10 cell *C. albicans* suspension and cells were lysed and DNA extracted as above and used as template for PCR. Fig 6.10 shows the PCR products obtained with the extracted DNA where it can be seen that two out of the six samples produced

a faint amplicon corresponding to the size of the *C. albicans* product while the larger contaminant amplicon observed before was present in five out of six samples. Nonetheless the products were analysed electrochemically and five out of six PCR products gave signals which ranged from 3.2nA to 30.3nA (the sample without any product gave a zero current). These values were again considerably lower than the signals produced by the PCR control (mean of 62.4 ± 7.2 nA).

The continued presence of the contaminant in the samples extracted from blood was a confounding factor in the results obtained with whole cell extraction products. It was noted that the contaminant only arose after the inclusion of the lysis step using lyticase buffer. The buffer had been prepared long before the sensitivity work was attempted and had been prepared in non-aseptic conditions for use in DNA extractions from large amounts of yeast and it was possible that the contaminant had entered the lysis buffer when it was first prepared but was never observed in PCR products with extracted DNA due to competition from the very large amounts of target yeast DNA that would have been present. A new batch of lyticase was used to make a fresh lysis buffer in aseptic conditions for the next attempt at extraction from 10 cell-spiked blood samples.

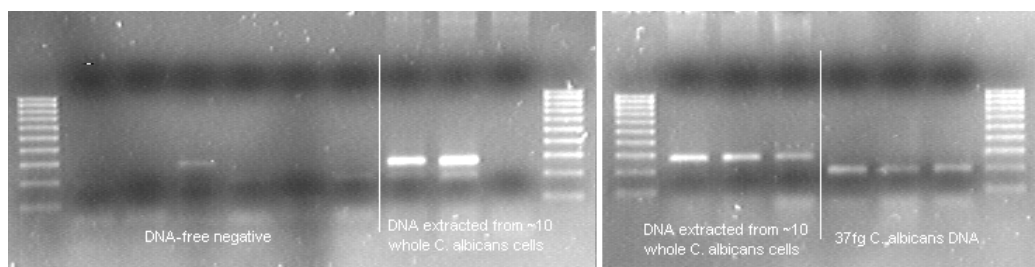


Fig 6.10. PCR products obtained using extracted DNA from 10 cell-spiked 1ml blood samples eluted in a smaller elution volume.

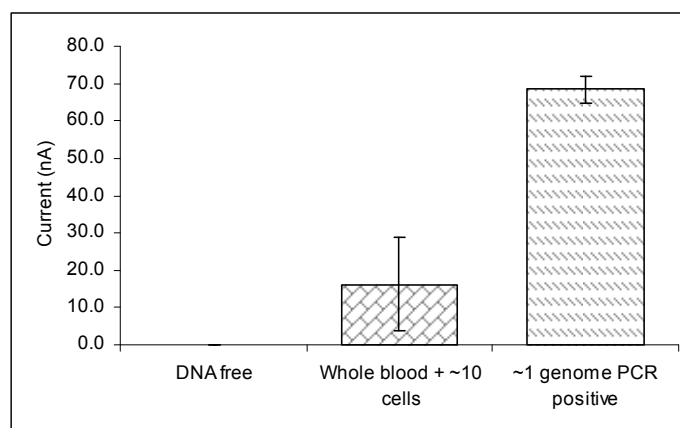


Figure 6.12. Mean currents obtained in assays using the PCR products shown in fig 5.11. Error bars represent SD (n=6 for DNA-free and spiked-whole blood samples, n=3 for PCR positive).

6.4.4. Detection of single *C. albicans* cells spiked into 1ml blood samples.

Reliable detection of 10 cells spiked into a 1ml blood sample was achieved previously and the conditions used to obtain that result were used to extract DNA from 1ml blood samples spiked with a *C. albicans* cell suspension containing 1 cell. In reality the blood was spiked with 10 μ l of a cell suspension that had been diluted to a concentration of 0.1 cells μ l⁻¹ and the actual number of cells added to the sample was liable to vary according to the Poisson distribution. Accordingly twelve 1ml blood samples were spiked with the cell suspension in order to ensure that a reasonable number of samples would actually receive one or more cells. DNA was extracted from blood and was used in PCR amplifications (fig 6.13).

Contamination of the DNA-free samples by two different targets was observed, including one that corresponded to the expected size of the *C. albicans* product. The amplicons produced by the extracted DNA showed contamination from several sources and only three out of the twelve reactions were contamination-free while only a single sample produced an amplicon of the expected size for *C. albicans*.

The samples were analysed electrochemically in spite of the contamination. None of the products from extracted DNA gave a signal indicating that the extraction procedure was not sensitive enough to achieve detection of 1 cell ml⁻¹ blood. The absence of cells in the blood samples was unlikely to be the cause for the lack of PCR product and consequent lack of current since the cell density of the suspensions was confirmed by plate counts which suggested that at least one sample would contain fungal cells. Other possible explanations include the loss of DNA during the extraction procedure due to its inefficient nature and the relatively large sample volume that must be handled – the columns are designed for centrifugation of only 700µl liquid at a time and a total of 2.25ml of sample had to be processed. Contamination was also still an important issue with the extraction procedure and persisted in spite of changes to some of the reagents but there was no way of knowing the source of the contaminants for sure, or the extent of their effect on the assay. Unfortunately, further experiments to extract and amplify DNA from 1 *C. albicans* cell were not able to be performed due to time constraints.

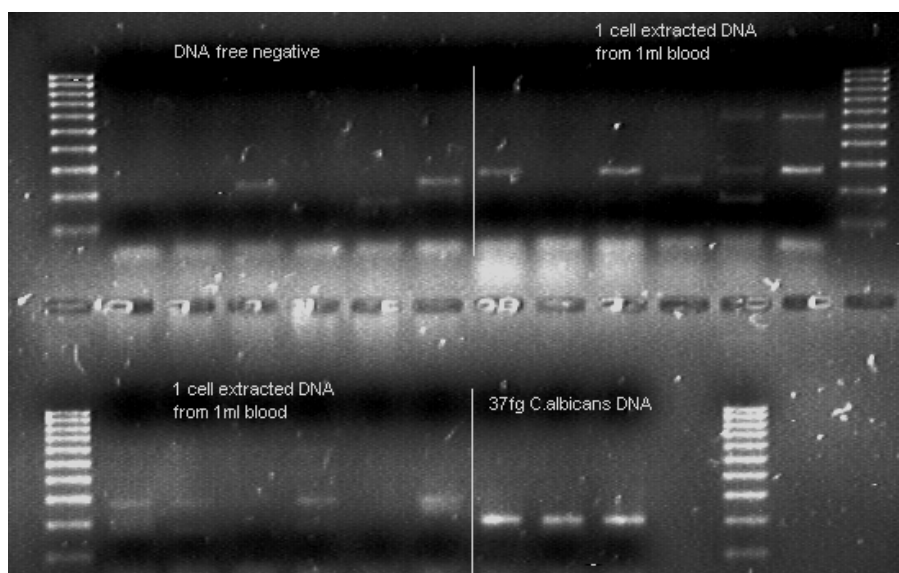


Figure 6.13. PCR products obtained using extracted DNA from 12x 1 cell-spiked 1ml blood samples.

6.5 Discussion and Conclusions

There was a difference in the values of current obtained when extracted *C. albicans* genomic DNA was spiked into blood, extracted and used as template compared to the currents produced by assays using equivalent amounts of *C. albicans* cells spiked into blood with the samples amplified from genomic DNA giving higher signals; most strikingly in the case of detecting 1 genome of extracted DNA in blood compared to detecting DNA from 1 cell in blood where the latter failed to give any signal whatsoever. There are a number of possible reasons as to why this would be the case. The most basic explanation might be that the concentration of genomic *C. albicans* DNA was underestimated leading to a larger amount of template being present in the reactions than thought. This could have happened when the initial concentration was measured by spectrometry or may have occurred due to user error when calculating and performing DNA dilutions to obtain a concentration of 37fg ul^{-1} . If time had allowed, replicated experiments with newly made DNA samples, or DNA samples from other *Candida* species, may have been able to lend support to this theory.

It was also observed that the currents produced by assays using product amplified from 1 genome of DNA spiked into blood gave lower signals than 1 genome added directly to a PCR reaction. This could have been due to DNA loss occurring during the extraction procedure, since such methods are never 100% efficient at recovering nucleic acid, or by the potential for PCR primers to be sequestered due to interactions with non-target DNA which would have been provided by the vast excess of human genomic DNA that would have been present in the extracted DNA sample.

The prototype nature of the extraction procedure was far from ideal, with many centrifugation steps being required to process each sample which may have been detrimental to the procedure due to increased risk of contamination and loss of nucleic acid, further reducing the efficiency of this column-based extraction method. This latter point was of particular importance when attempting to detect DNA from a single *C. albicans* cell in a 1ml blood sample.

It was possible that the extraction procedure could have been further optimised for recovery of small amounts of fungal DNA. However, any modifications to the protocol would have to be technically simple to maintain the ease of use of the system. An obvious potential modification to the procedure to allow detection of a single cell would have been to centrifuge the whole blood to pellet the cell fraction; the plasma could then be removed and the procedure carried out as normal resulting in smaller volumes being handled and fewer centrifugation steps, so reducing the potential for DNA loss. This change combined with the use of smaller volumes of lysis buffer may have allowed the procedure to operate with only a single centrifugation step, enabling detection of a single *C. albicans* cell and improved detection of larger numbers of cells, as well as vastly increasing the turnaround time of experiments.

Although discussion of how the final assay might work based on Atlas Genetics' portable Velox system is ultimately beyond the scope of this work, it is pertinent to note that minimal sample handling is required prior to testing, and use of a relatively small sample size is a prerequisite for the system. Quite how the detection of, for example, 25 cells in a 5ml blood sample would be achieved using a 200µl fraction for analysis with little or no sample preparation is hard to imagine.

While spiked blood samples from a healthy patient are useful for preliminary testing of a detection assay's capability the use of clinical samples would provide much more definitive results. Samples taken from patients with an episode of candidemic infection may differ from healthy blood samples, particularly in terms of the leucocyte cell population and can also provide a better measure of the sensitivity of an assay – indicating the likelihood of detection of fungal DNA from a sample taken under clinical conditions. Unfortunately the acquisition of such samples is no easy task and usually requires a prospective request for appropriate samples to be taken from candidaemic patients and this was not possible due to time constraints.

Nonetheless, the assay was shown to be sensitive enough to detect 1 genome of extracted *C. albicans* DNA per millilitre of blood but was less sensitive when intact cells were used, achieving a limit of detection of only 10 cells per millilitre of blood. This detection limit is within the clinical range for candidaemia of 5 to ≥ 100 c.f.u ml⁻¹ (Loeffler *et al.*, 2000) but a lower limit outside of the range would be preferable to avoid the possibility of false negative results. It was also found that the type of blood sample made little difference to the results - there was no significant difference between the currents obtained when spiked whole blood samples were assayed compared to spiked plasma samples when relatively large amounts of *C. albicans* genomic DNA were used.

Unfortunately time did not permit the testing of the limit of detection of the assay with the other *Candida* species and their probes. Given the previous work performed on the probe suite it can be tentatively assumed that a similar limit of detection of 10 cells per ml of blood would be achievable by all of the other probes given minor optimisation, with the possible exception of the *C. krusei* specific probe CK PR, but experimental confirmation of this is, of course, an absolute necessity.

Chapter 7. Further Observations on the Assay Model

7.1 Introduction

The general model of the assay was outlined in chapter 3 but the full workings have not been established. While developing the fungal probe suite the opportunity occasionally arose to study different aspects of how the electrochemical assay produced signals in the presence of target DNA. Some of this work was presented in chapter 3 by way of an introduction to the assay and to furnish the reader with the necessary background information to understand it and the results that it produced.

This chapter presents further observations that were made concerning the assay which increase the understanding of the assay mechanisms and which may be of potential importance should additional optimisation be required to improve the results generated.

7.2. Effect of the reaction medium on assay signal.

It had previously been established that diffusion was an important factor in production of the assay signal. Therefore factors which affect diffusion should also have an effect on the current produced by the assay. One factor which was examined was the composition of the reaction medium through which an electroactive molecule must move. In the assay this medium is the post-PCR reaction mix, composed of various buffer components, salts, nucleotides, primer-dimer oligonucleotides and left-over *Taq* DNA polymerase supplemented by T7-exonuclease and its storage buffer components. According to the diffusion theory model, a more dilute reaction medium would allow larger molecules to move more rapidly through it. This would result in the background current increasing to the same level as that produced by target containing samples and this could be tested with the assay.

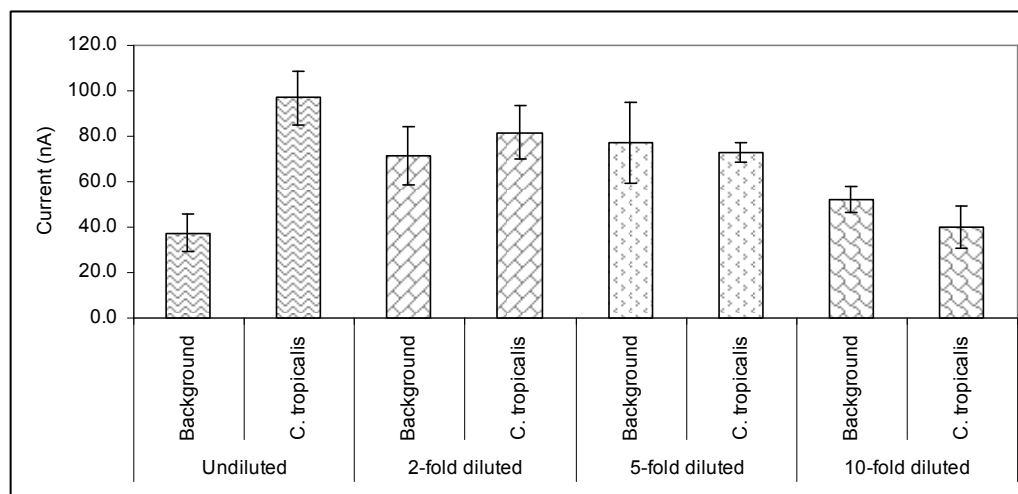


Figure 7.1. Mean currents obtained in assays with CT PR using PCR products diluted with H₂O. Error bars represent SD (n=4).

PCR products were diluted 2, 5 and 10-fold with H₂O before incubation with probe and T7 exonuclease. The undiluted samples produced results as expected (fig 7.1) with a mean background current of 37.4 ± 8.2 nA compared to a positive signal 96.8 ± 11.9 nA. When the PCR products were diluted 2-fold the signal obtained with the positive sample decreased slightly to 81.8 ± 11.9 nA while the background signal increased by almost double to a value of 71.3 ± 12.9 nA. At a five-fold dilution the positive signal and background signal were of a similar level with mean currents of 72.9 ± 4.5 nA and 76.9 ± 19.0 nA respectively and at a 10-fold dilution both samples exhibited a decrease in current which was less marked in the background sample which gave a signal of 53.0 ± 5.7 nA compared to a positive signal of 39.9 ± 9.2 nA.

The results demonstrated that diluting the reaction medium with H₂O had two noticeable effects on current production. Firstly, there was an approximately linear decrease in the signal produced by the target-containing positive samples which fell from 96.8 ± 11.9 nA to 39.9 ± 9.2 nA; secondly, the background current exhibited an increase to similar levels as the positive samples at each dilution with the background current rising from its initial level at two- and five-fold dilutions before decreasing in a similar way to the positive sample current at 10-fold dilution.

These results provided some support for the theory that dilution of the reaction matrix allows increased diffusion of larger molecules to the electrode but also indicated that dilution was causing an overall decrease in signal at higher dilutions. This was possibly due to the dilution of ionic species in the reaction mix which are involved in transfer of electrons and this would have inhibited electron transfer at the electrode leading to smaller currents. In order to try and negate this effect another dilution experiment was set up with dilution of PCR product carried out using 1X PCR buffer in order to maintain the concentration of ions in the reaction medium. The dilutions were also made over a larger range, from a 10-fold dilution to a 10^4 dilution.

The results (fig 7.2) showed the predicted increase of the background signal when the samples were diluted but did not exhibit the decrease in positive and background currents that had been observed at higher dilutions when H₂O was used as the diluent. The mean current produced by target samples remained significantly higher than the background up to a 100-fold dilution. At 1000-fold and 10000-fold dilutions the mean currents produced by the background and positive samples differed by only 2 - 4nA.

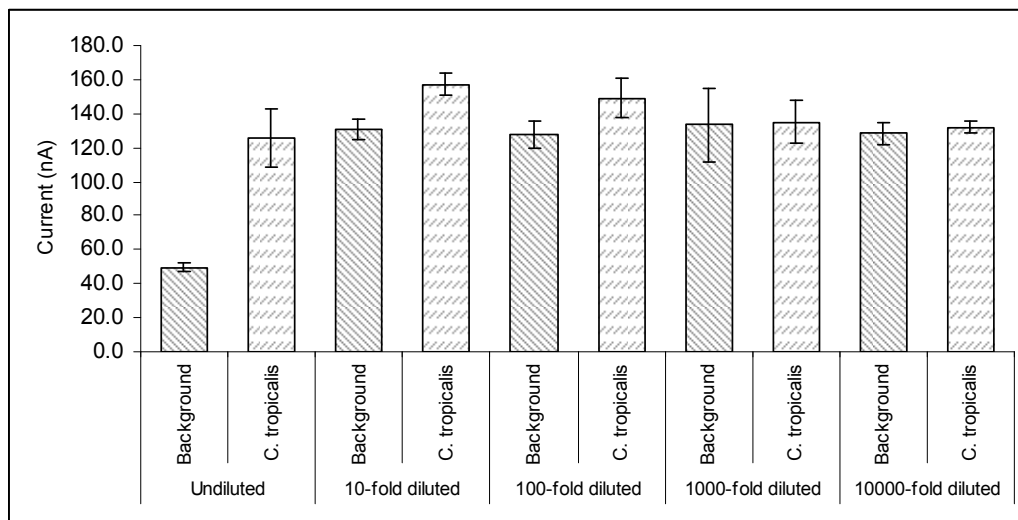


Fig 7.2. Mean currents produced by assays using PCR product diluted with 1X PCR buffer. Error bars represent SD (n=4).

The effect of adding different amounts of Tween 20 to the reaction mix was tested to see if the reverse effect to that described above could be observed, whereby increasing the complexity of the reaction matrix could lead to a decrease in signal by reducing the ability of molecules to diffuse through it. The experiment was conducted using the now redundant CL PR probe which was designed to detect DNA from *C. lusitaniae* and the results are shown in figure 7.3 where it can be seen that there was a general decreasing trend of the mean current of both signal and background as the amount of Tween 20 present in the reaction increased. The target-containing samples produced a mean current of $60.0 \pm 8.4\text{nA}$ in the presence of 0.01% Tween which decreased almost twofold to a value of $32.1 \pm 2.6\text{nA}$ when the concentration of Tween was 0.5%. The background signal decreased almost ten-fold, from a value of $11.6 \pm 2.9\text{nA}$ at 0.01% Tween to $1.8 \pm 0.2\text{nA}$ at 0.5% Tween, demonstrating that Tween was having an inhibitory effect on the production of signal in the assay. Despite the reduction in assay signal attributable to the presence of Tween there was evidence that use of such a surfactant could be used to improve the signal:background ratio.

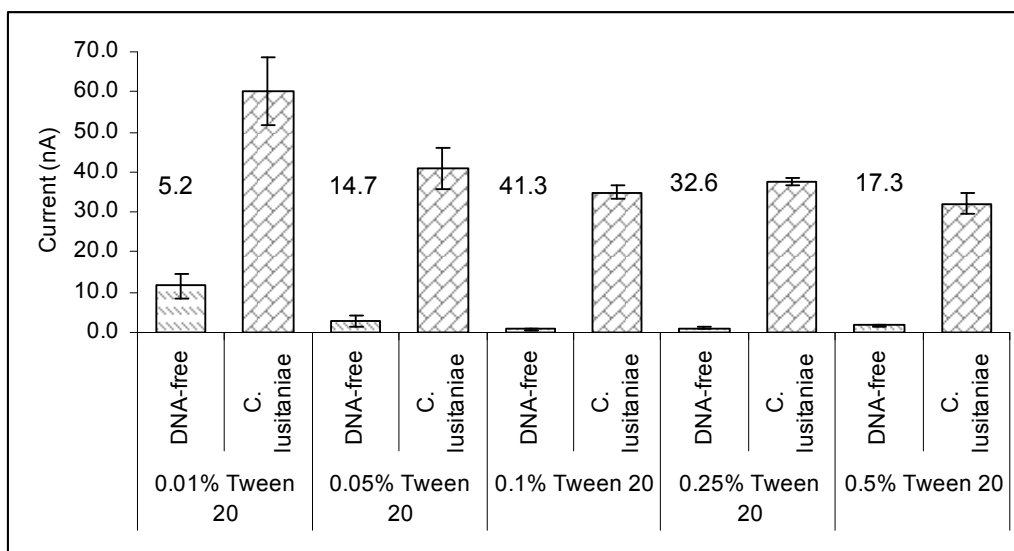


Figure 7.3. Mean currents produced in assays with probe CL PR in the presence of increasing amounts of Tween 20. Ratios of signal:background are also shown. Error bars represent SD (n=4).

While the results by no means conclusively proved that diffusion was the key assay process being affected, they did provide strong support for the theory that diffusion of electro-active molecules to the electrode surface was a major factor in production of current in the assay and allowed differentiation between the background signal produced by undigested probe and the positive signal produced by probe digested in the presence of target DNA and T7 exonuclease. They provided a degree of support for the hypothesis that the zero-background results obtained when using the Jumpstart Taq were due to the presence of surfactants in the enzyme storage buffer. The results of the dilution experiments demonstrated that diluting out of ionic species with H₂O can lead to a loss of current which, while not necessarily an important result in itself, does highlight the fact that there are numerous complex processes occurring in the reaction which interplay together to produce the assay signal and trying to investigate a single one in isolation is not always easy.

7.3 Probe stability

The probes were all stored at -20°C and used frequently, some for over 2 years, and only the CK PR probe displayed a considerable reduction in the value of current that it produced. It was not possible to draw direct comparisons between the results obtained in the initial stages of the project and the final stages as the assay had changed in between, but figure 7.4 demonstrates that the oldest probes were still capable of providing a satisfactory signal (which was actually much improved in some cases, thanks to an improved method and better designed primers) over 2 years after they were first acquired. The results are not directly comparable as they were performed on PCR products using different amounts of DNA – nanogram vs. picogram amounts – and different PCR conditions. Despite an approximately 1000-fold reduction in the amount of template the observed decrease in signal between nanogram amounts of *C. tropicalis* DNA amplified using Illustrataq polymerase with ITS3/ITS4 conditions compared to picogram amounts of DNA

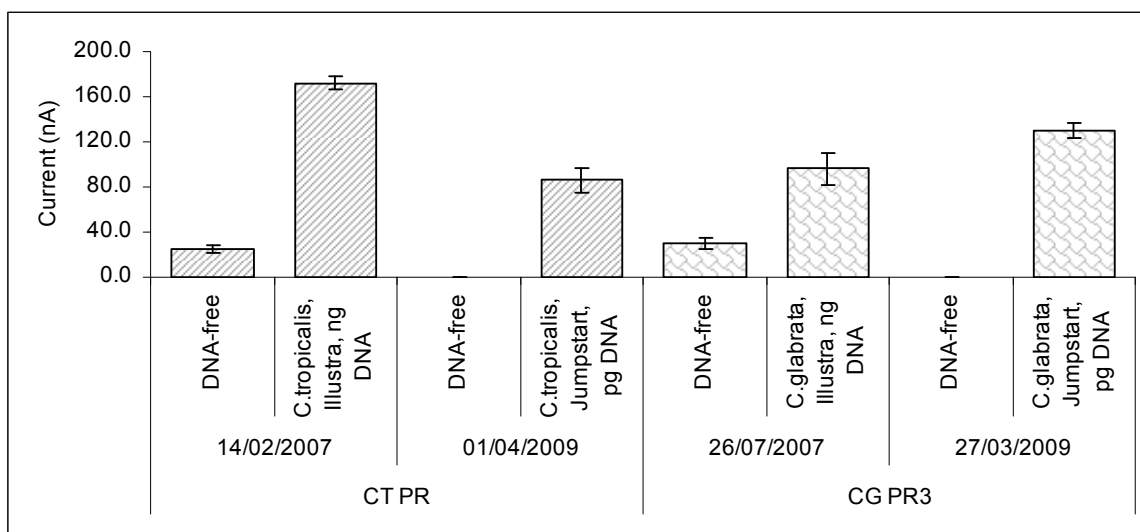


Figure 7.4. Mean currents produced by assays using CT PR and CG PR3 probes on different dates to demonstrate the effect of long-term storage on probe stability. Error bars represent SD (n=4 for the earlier assays and n=3 for the later assays).

amplified using Jumpstart polymerase and ITS3.3/ITS4.2 was only 50%, from 172.3 ± 6.2 nA to 86.3 ± 11.1 nA. The signal obtained with picogram amounts of *C. glabrata* DNA amplified using Jumpstart polymerase and ITS3.3/ITS4.2 actually showed an increased signal compared with the signal obtained using nanogram amounts of *C. glabrata* DNA amplified using Illustrataq and ITS3/ITS4 – from 96.1 ± 14.1 nA to 130.7 ± 6.5 nA.

It was difficult to explain why the signal obtained with the CT PR probe should decrease across the two assays while the signal obtained with the CG PR3 probe actually increased. Part of the problem was that the properties of probes seemed to depend on a number of factors which meant that each probe had its own unique characteristics in terms of the signal produced with different reagents and DNA amounts. This may have been for a range of reasons including batch differences when the ferrocene label was attached by ATDBio and differences in the probe sequence which may have affected the assay. The latter point, in my opinion, is a very important issue as phenomena such as secondary structure formation or the stoichiometry of different combinations of base pairs may affect the production of signal and

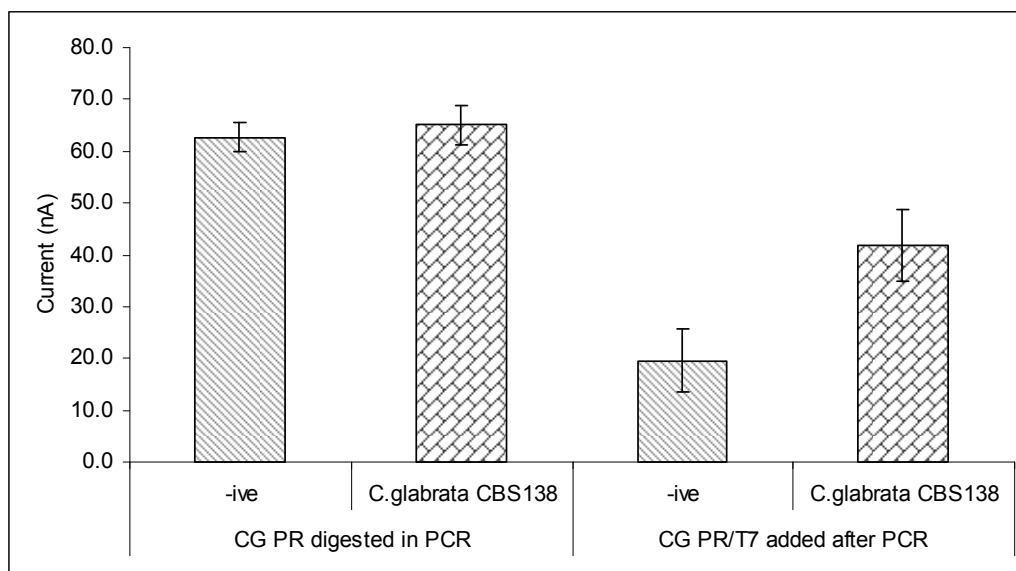


Figure 7.5. Mean currents produced in assays using probe added directly to PCR and digested using *Taq* polymerase vs. assays using products amplified in the same way but digested post PCR.

therefore further inform design of reliable probes. While these theories were testable, due to the high cost of reagents the required experiments could not be performed in the course of this work.

The heat stability of the probe was also the subject of experimentation. An obvious question was the possibility of performing the probe binding and digestion steps within the PCR reaction in exactly the same way as a Taqman assay. Figure 7.5 shows the currents that were generated from an assay in which the probe CG PR was added to the PCR mix and samples amplified in a Roche lightcycler using cycling parameters developed for Taqman assays. A control consisting of the same PCR mix without probe CG PR was used to amplify samples simultaneously, and the probe and T7 exonuclease were added post-PCR to produce electroactive products for analysis. The background current produced by samples containing probe that had been exposed to PCR conditions gave an unusually high signal of $62.6 \pm 2.9\text{nA}$ compared to a background signal of $19.5 \pm 6.1\text{nA}$ produced by the control samples ($P < 10^{-5}$). It was also highly significantly greater than the $41.9 \pm 7.0\text{nA}$ signal produced by the target-containing control sample ($P < 0.005$). The mean current produced by the target-containing samples which had

probe added to the PCR reaction was also relatively high at $65.1 \pm 3.7\text{nA}$ and was significantly higher than the signal produced by the target-containing control ($P < 0.005$) but was not significantly different to the background signal produced when probe was present in the PCR.

The similarly high values of current produced by both the target and non-target samples with CG PR added to the PCR suggested that a similar amount of oxidation was occurring in these samples and it was greater than when the probe was digested post-PCR by T7 exonuclease at 37°C . A possible explanation for this was that the bond between the ferrocene label and the probe was not heat stable and the high temperatures experienced during PCR cycling were allowing release of the label from all of the probe molecules regardless of the presence of target DNA.

This theory was tested by adding probe CG PR to 1X PCR buffer and subjecting the samples to the PCR conditions used above while other samples were kept at room temperature for the duration of the program. Electrochemistry readings were then taken from each sample type and the mean currents are shown in figure 7.6. The samples incubated using the PCR parameters gave a signal of $171.2 \pm 12.0\text{nA}$ which was very highly significantly greater than the signal obtained with the probe incubated at room temperature: $128.8 \pm 8.0\text{nA}$ ($P < 0.001$). This demonstrated that simply heating the probe led to an increase in current meaning the assay could never be adapted to be used in the same way as Taqman or other related assays. It would have been interesting to perform the experiment again, but with the caveat of preparing two sets of PCR mixes, one with and one without probe. Both sets would then be heated using the assay's PCR parameters and probe added to the probe-less mix afterwards and electrochemical measurements taken. Such an experiment would have given results that were more likely to reflect other factors present in the assay such as the reaction matrix and presence of other oligonucleotide molecules.

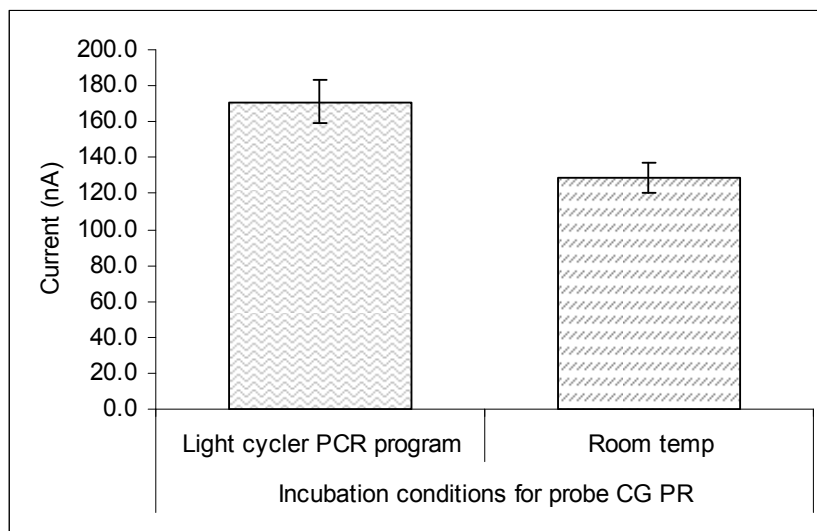


Figure 7.6. Mean currents produced by PCR-cycled probe vs. probe incubated at room temperature.

7.4 Using whole yeast cells as template for the assay

Experiments into the effects of having lysed yeast cell debris present in the assay were performed alongside comparative experiments using quantified numbers of whole cells added directly to PCR and extracted DNA from the same numbers of yeast cells as template. Figure 7.7 shows the mean currents produced in assays using PCR products which were amplified from the following sources: excess extracted *C. krusei* DNA which was then spiked with cell lysate following PCR, known numbers of whole *C. krusei* cells or DNA extracted from known numbers of *C. krusei* cells. The presence of cell lysate from 500 cells did not have a statistically significant effect on the mean current produced in the assay compared with the mean current produced by the samples with *C. krusei* DNA alone but this was very likely due to an outlying result produced by the latter samples which resulted in a relatively high standard deviation. The mean current produced by DNA + 500 cells lysate was significantly lower than the mean current produced by DNA + 100 cells lysate ($P < 0.05$) and DNA + 50 cells lysate ($P < 0.01$). There was no

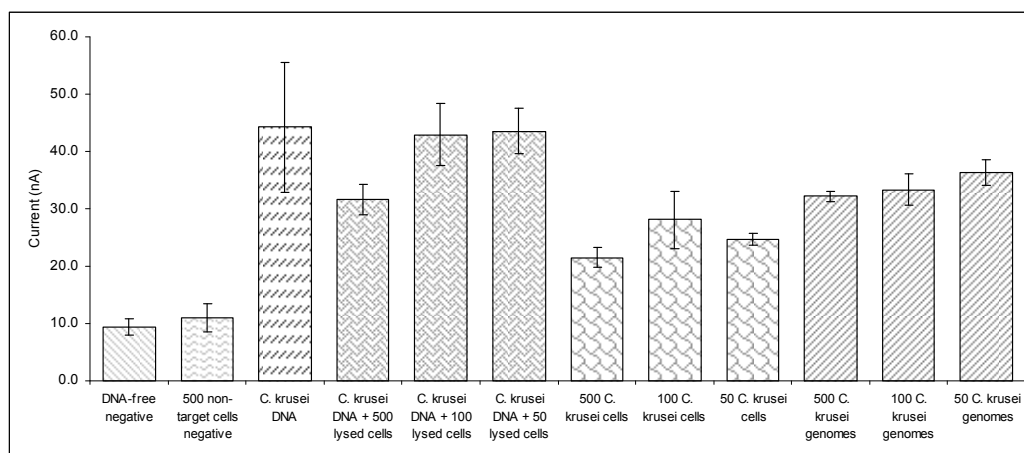


Figure 7.7. Mean currents produced in assays testing the effect of yeast cell lysate on current production using the probe CK PR. Error bars represent SD (n=3).

significant difference between the mean currents produced when lysate from 100 or 50 cells was present in assays with excess DNA. These results suggested that the presence of large amounts of lysate (≥ 500 cells) was having a detrimental effect on the current production in the assay.

When whole cells were used in the assay compared to DNA extracted from the same number of cells there was a very highly significant difference between the mean currents produced by 500 cells vs. 500 genomes ($P < 0.0005$) and 50 cells vs. 50 genomes ($P < 0.001$) however there was no significant difference between the signals produced by 100 cells vs. 100 genomes and this was again probably due to the presence of an outlying current which produced a relatively high standard deviation for the samples with 100 cells.

These data indicate that, generally speaking, whole cells produce lower signals in the assay compared to a comparable amount of extracted DNA. There are a number of possible reasons why this would be the case such as incomplete lysis of cells during PCR leading to reduced amounts of template DNA or inhibition of *Taq* DNA polymerase or T7 exonuclease by the fungal cell debris could also account for the difference in signal. These findings may explain why it was not possible to obtain a PCR product from 10 *C. albicans* cells added directly to PCR (see chapter 6).

7.5 Conclusions

There are many factors which contribute to the current produced in a given assay. Some of these, particularly the factors pertaining to the PCR stage of the assay, are relatively well understood and the reasons for their effect on the assay can usually be explained thanks to the body of knowledge that underpins such a well-used and well-studied methodology. However, the electrochemical portion of the assay is much less well understood as it is a novel methodology with only a handful of people involved in its study. The basic model of the assay, hybridisation of a labelled probe which is then digested to release a labelled mononucleotide, was introduced in chapter 3 and is a fairly well known concept as it is frequently used with fluorescent detection procedures but it is the method of detection by electrochemical means that makes the system unique and less easily understood.

The findings discussed here and in chapter 3 shed some light on some of these less well understood areas and offer refinements to the basic model of electrochemical detection by ferrocene-labelled probes. Unfortunately since the experiments were never part of a focused project to elucidate how different features of the assay work, the results are far from conclusive but amount to a small, but significant, body of evidence in support of these refinements. Of particular interest were the results of the experiments into the various factors relating to the probes as this type of labelled probe is unique to Atlas Genetics and has not been exhaustively studied. The conclusion that diffusion is a highly important factor in the production of current demonstrates further how the assay differs from conventional fluorescence based assays where the presence of a released fluorescent molecule in a reaction mix is sufficient to contribute to the signal as opposed to the electrochemical assay in which the released label must also make its way to an electrode surface to contribute to the output.

The major drawback to the above conclusions is that there is nothing fundamental that supports and validates them - they were all produced using the assay which they then make predictions about. It would be informative to

perform further experiments into diffusion of electroactive molecules, for example, using an unrelated system to validate the conclusions made here.

Chapter 8 – Discussion & Conclusions

8.1 Discussion.

Using the electrochemical detection method established by Atlas Genetics a suite of six probes, capable of detecting DNA from fungi and of identifying five of the most clinically relevant species, was developed. Using an improved PCR protocol, all of the probes were demonstrated to be able to detect DNA from target species to a level of ~1 genome present in a 50µl reaction volume and give zero background signal. The specificity of the probes was confirmed experimentally against a test panel of nine non-target *Candida* species and against these nine species plus a further 27 fungal species when examined *in silico*. A strategy of rapidly screening probe sequences against a matrix of fungal ITS2 sequences using the BLAST algorithm was also developed and was used to screen newly designed probes for specificity, as well as support the panfungal nature of the PanF PR probe. The robustness of the species-specific probes was also confirmed in tests with wild strains of the relevant species.

The pan-fungal probe was designed to be able to detect a wide range of fungi including emerging opportunistic fungi, such as *Rhizomucor*, *Absidia*, *Fusarium* and *Penicillium* species, as well as the more commonly occurring *Cryptococcus*, *Aspergillus* and *Candida* species. It successfully detected 14 species of opportunistic fungal pathogens including several infrequently encountered species, and one non-pathogenic fungal species, *Cr. victoriae*. The probe was also shown to be highly likely to be able to detect DNA from a total of 26 fungal species examined *in silico*, including some of the emerging pathogenic fungi.

Finally, the *C. albicans* specific probe was shown to be capable of detection of ~1 genome of extracted DNA spiked into a 1ml blood sample or 10 c.f.u. of *C. albicans* spiked into the same volume of blood following the application of a relatively straightforward total DNA extraction procedure which was developed for the purpose.

The probe set was designed to detect DNA from any fungal pathogen, as well as to achieve species-specific detection of the most common species involved in invasive fungal infections including *C. glabrata* and *C. krusei* which are associated with decreased susceptibility to fluconazole. Detection methods that permit species-specific detection of the most common fungal pathogens, including fluconazole resistant species, are generally unsatisfactory as they cannot detect infecting species outside the test panel and the majority of methods of detecting invasive fungal infections have concentrated on a few common fungi, usually *Candida* species (Shin *et al.*, 1999; Maaroufi *et al.*, 2003; Basková *et al.*, 2007; Innings *et al.*, 2007; Metwally *et al.*, 2007). Only a few methods have attempted to achieve detection (but not identification) of a range of fungal species (e.g. Vollmer *et al.*, 2008) and very few have tried to design a probe capable of detecting all pathogenic fungi (e.g. Zhao *et al.*, 2009). The inclusion of a pan-fungal probe in this suite means that early detection of an invasive infection caused by any fungal pathogen could be achieved enabling a patient to be monitored and treated more successfully. A potential drawback to the use of a highly sensitive panfungal probe is that it necessitates extremely careful handling of samples since detection of even very low amounts (10 cells) of contaminating fungi from the environment may be detected by the assay.

The limit of detection of the assay in blood was within a diagnostic range but was not as good as that achieved by other molecular detection methods (e.g. White *et al.*, 2003; Klingspor & Jalal, 2006; Basková *et al.*, 2007). The limit of detection may have been negatively affected by the relatively unsophisticated method of total DNA extraction from spiked blood - there were many opportunities for sample loss due to multiple centrifugation steps and the inability to use 100% of the lysate since the dregs could not be removed from the original vial and added to the spin column. A study by Metwally *et al.* (2008) demonstrated that the choice of DNA extraction procedure can make a significant difference to the sensitivity of a diagnostic assay and it is likely that had the extraction procedure been improved further it would have resulted in increased signals at low cell densities and an improved sensitivity with the electrochemical assay. The same study also suggested that contamination of

PCR by human rRNA may act to sequester primers. This was pertinent since the human 5.8S and 26S rRNA genes have target sites which are similar to the binding sites of the ITS3.3/4.2 primers used with the electrochemical assay. These rRNA target sites could only produce short, ssDNA molecules but in doing so could have depleted the primers which would have had a detrimental effect on the assay sensitivity. The presence of human DNA in PCR may also have had an effect of the efficacy of amplification of the fungal ITS2 target due to random sequestration of the primers to non-specific target sites, as well as the genes coding for rRNA.

As the work on the limit of detection in blood was begun with only a few months until the end of the project the limit of detection could only be ascertained for the *C. albicans* specific probe. Since this was the best performing probe in the initial limit of detection work it would not be appropriate to assume that the other probes would behave similarly and so it is not known if the other probes would have been able to achieve the same limit of detection of *Candida* DNA or cells in blood samples. The *C. krusei* specific probe in its current form would almost certainly not be capable of detection of 10 cells ml⁻¹ blood and so it is likely that a redesign of this probe and further optimisation of the DNA extraction procedure would be required to achieve a satisfactory LOD for the remaining probes.

Detection of *Candida* from clinical samples is the truest validation of a diagnostic assay but unfortunately such samples could not be obtained in time. Following confirmation of the LOD of all of the members of the probe suite, successful testing of the assay using clinical samples containing cells of the appropriate species must be performed to show that the method is suitable for such a purpose.

A further problem that affected the limit of detection work in PCR reactions and from blood was that of contamination. There were two likely sources of the contamination observed in the work: the first was the environment - fungi are ubiquitous organisms and the lab in which the work was conducted is situated in close proximity to woodland with much decaying material. The lab

also featured an air conditioning system which would have allowed access to the lab environment for airborne cells and spores. The other source was amplified target DNA released into the lab environment following post-PCR manipulation of reaction products. The presence of contaminating material was made more problematic by the fact that fungal universal primers were used which meant that DNA from any species of fungi was likely to be amplified. The problem was somewhat alleviated by setting up reactions in a class 2 biological safety cabinet which was routinely sterilised by UV irradiation, however the contamination issue could not be completely removed. Methods for prevention of PCR amplicon contamination exist (e.g. Longo *et al.*, 1990; Rueckert & Morgan, 2007) and implementation of contamination control is necessary for work involving repeated amplification of the same PCR target, particularly when detection of low number of target molecules is being attempted.

The capability of multiplex detection would be an extremely useful feature of the probe suite and the probes have been designed with this in mind. The use of a single pair of fungal universal primers means that the likelihood of primer dimerisation is reduced and there is no chance of non-target amplification due to 'unassociated' primers binding to non-specific target sites by chance. Furthermore, fungal universal primers allow the amplification of any fungal DNA present and specific detection can then be made by the species-specific probes. This feature allows the easy incorporation of further species-specific probes to the suite. Multiplex detection would be achieved by the use of ferrocene labels with different oxidation potentials. In a similar way to fluorescent labels which emit light at different wavelengths, ferrocene molecules can be designed which are oxidised at different voltages and this would allow differentiation between which probes are producing a signal. Work performed by J. A. Olds with Atlas Genetics demonstrated that multiplex detection utilising three probes labelled with different ferrocene molecules was possible (unpublished data). However, to date the potential for multiplex has been limited by unavailability of robust ferrocene probes with different oxidation potentials. Work is ongoing within the Dept of Chemistry at the University of Bath to synthesise these molecules reproducibly.

8.2 Conclusions.

The assay which was developed in this work has the potential to be developed to provide frontline rapid diagnosis of invasive fungal infections. No single technique is flawless in its ability to diagnose invasive fungal infections since traditional culture-based methods are slow and not always specific while rapid molecular methods require trained personnel, specialised equipment and do not necessarily prove invasive fungal infection since they detect nucleic acid. Therefore, a synergistic approach to detection and identification of fungal pathogens would offer many advantages over reliance on a single 'gold standard' method. The rapid molecular techniques offer an early warning system for suspected fungal infections and can also indicate potentially drug resistant species so that appropriate therapy can be initiated at once. Further confirmation of the presence of a fungal pathogen can then be obtained by tests for fungal antigens and culture of fungi from patient samples. This approach would ensure that patients receive optimum antifungal therapy and the costs of treatment are minimised as there is less chance of inappropriate therapy being administered.

A major problem with this approach is that not all hospitals have access to the same equipment and expertise, particularly where rapid molecular tests are concerned. The Atlas Genetics proposed at-or-near point-of-care device aims to resolve this issue by providing a generic platform for detection of pathogens from patient samples with minimal user input thus providing a method of rapid molecular detection of pathogens to all hospitals.

The results obtained show that the described methodology and probes have the required specificity, and could obtain the necessary limit of detection, to detect fungal DNA from samples and identify to species level the most clinically relevant fungi. This key work advances the position of the fungal detection suite so that it is closer to being adapted and miniaturised for use in Atlas Genetics' proposed at-or-near point-of-care format, and would be an excellent early detection system for fungal pathogens requiring minimal user expertise and facilities.

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